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Uncovering the hepatotoxic and neurotoxic potential of β-keto amphetamine (cathinone) derivatives:

in vitro mechanistic studies

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Aos meus pais
Ao meu irmão Zé
À Detinha
Ao Luís

Publications

In accordance to the nº 2, paragraph a, article 31 from Decree-Law nº 115/2013, the following published/submitted articles and conference papers were prepared under the scope of this thesis.

Articles published or submitted to international peer-reviewed journals

I. Valente MJ, Guedes de Pinho P, Bastos ML, Carvalho F, Carvalho M (2014) Khat and synthetic cathinones: a review. *Arch Toxicol* 88(1):15-45 doi:10.1007/s00204-013-1163-9

II. Valente MJ, Araújo AM, Silva R, Bastos ML, Carvalho F, Guedes de Pinho P, Carvalho M (2016) 3,4-Methylenedioxypyrovalerone (MDPV): *in vitro* mechanisms of hepatotoxicity under normothermic and hyperthermic conditions. *Arch Toxicol* 90(8):1959-73 doi:10.1007/s00204-015-1653-z

III. Valente MJ, Araújo AM, Bastos ML, Fernandes E, Carvalho F, Guedes de Pinho P, Carvalho M (2016) Characterization of hepatotoxicity mechanisms triggered by designer cathinone drugs (β-keto amphetamines). *Toxicol Sci* 153(1):89-102 doi:10.1093/toxsci/kfw105

IV. Valente MJ, Bastos ML, Fernandes E, Carvalho F, Guedes de Pinho P, Carvalho M (2017) Neurotoxicity of β-keto amphetamines: deathly mechanisms elicited by methylone and MDPV in human dopaminergic SH-SY5Y cells. *ACS Chem Neurosci* doi:10.1021/acschemneuro.6b00421

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Conference papers in international peer-reviewed journals

I. Valente MJ, Araújo AM, Carvalho F, Bastos ML, Guedes de Pinho P, Carvalho M (2014) An insight into the mechanisms underlying the hepatotoxicity of cathinone derivatives. *Toxicol Lett* 229:S58. doi:10.1016/j.toxlet.2014.06.235

II. Valente MJ, Araújo AM, Silva R, Bastos ML, Carvalho F, Guedes de Pinho P, Carvalho M (2015) Hepatic oxidative stress induced by methylone and MDPV: A comparison to MDMA. *Toxicol Lett* 238(2):S265. doi:10.1016/j.toxlet.2015.08.765

III. Valente MJ, Araújo AM, Silva R, Bastos ML, Carvalho F, Guedes de Pinho P, Carvalho M (2015) Is hyperthermia the triggering factor for hepatotoxicity induced by 'bath salts'? An in vitro study using primary cultured rat hepatocytes. *Toxicol Lett* 238(2):S260. doi:10.1016/j.toxlet.2015.08.752

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Author's declaration

The author declares to have actively contributed to the conception and technical execution of the work, acquisition of data, analysis and interpretation of the results, as well as to the manuscript preparation of the published or submitted work included in this thesis, in close collaboration with all other authors.

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Resumo

As catinonas sintéticas, comummente designadas por β-ceto-anfetaminas, são substâncias psicostimulantes que surgiram no panorama de consumo recreacional durante o século XXI, sendo consumidas como alternativa a drogas de abuso ilícitas, como as anfetaminas e a cocaína. Ao longo dos últimos anos foram reportados vários casos de intoxicações e mortes associados ao consumo de β-ceto-anfetaminas. Os efeitos adversos incluem agitação severa, hipertensão, taquicardia, hipertermia, paranóia e psicose aguda, bem como danos em vários órgãos, tais como cérebro, fígado, rins, coração e tecido muscular. No entanto, os mecanismos subjacentes à toxicidade nos órgãos-alvo ainda não foram totalmente compreendidos. Considerando as semelhanças químicas e farmacológicas entre as catinonas sintéticas e as anfetaminas clássicas, postulou-se a existência de mecanismos toxicológicos comparáveis.

O primeiro objetivo desta tese passou pela avaliação *in vitro* do potencial hepatotóxico de quatro catinonas frequentemente consumidas, nomeadamente a 3,4-metilenodioximetcatinona (metilona), a 3,4-metilenodioxipirovalerona (MDPV), a pentedrona e a 4-metiletcatinona (4-MEC), em culturas primárias de hepatócitos de rato e células HepaRG diferenciadas, num estudo comparativo com a já extensamente estudada e hepatotóxica 3,4-metilenodioximetanfetamina (MDMA). Os resultados obtidos mostraram que todas as β-ceto-anfetaminas induziram morte celular em ambos os modelos *in vitro* de uma forma dependente da concentração. Os estudos mecanísticos conduzidos em culturas primárias de hepatócitos de rato suportam a capacidade das β-ceto-anfetaminas de induzir hepatotoxicidade *in vitro* através do aumento do stress oxidativo, da perturbação das funções mitocondriais e da indução de morte celular por apoptose. A MDPV e a pentedrona foram as substâncias com maior potencial hepatotóxico, sendo mais tóxicas do que a própria MDMA, enquanto a metilona exibiu um menor potencial hepatotóxico. De destacar a correlação direta verificada entre o potencial tóxico das catinonas e as suas características lipofílicas. Foi observada ainda uma redução parcial da morte celular na presença do inibidor da CYP2D6, quinidina, resultados que salientam a importância da bioativação metabólica na hepatotoxicidade das catinonas sintéticas.

A hipertermia é um dos efeitos agudos inerentes ao abuso de β-ceto-anfetaminas. Os resultados obtidos mostraram que o aumento da temperatura por si só interfere com o estado redox intracelular, observando-se também o agravamento de todos os efeitos hepatotóxicos da MDPV em condições hipertérmicas, caracterizado pelo aumento da produção de espécies reativas de oxigénio e azoto, depleção de glutationa e ATP, e aumento dos níveis de cálcio intracelular. A morte celular por apoptose foi precipitada pelo

aumento da temperatura de incubação, com um aumento da prevalência de necrose celular a concentrações mais elevadas de MDPV.

Foi igualmente avaliado o potencial neurotóxico *in vitro* da metilona e da MDPV em células SH-SY5Y dopaminérgicas. Verificou-se que ambos os derivados induzem morte neuronal de forma dependente da concentração, com aumento da produção de espécies reativas de oxigénio e azoto e depleção da glutationa, bem como disfunção mitocondrial, caracterizada pela despolarização da membrana mitocondrial e depleção de ATP. A indução de morte celular por apoptose foi também um mecanismo comum a estas duas catinonas, observando-se a ativação de mecanismos intrínsecos e extrínsecos, formação de núcleos picnóticos, condensação de cromatina e prevalência de células num estadio avançado de apoptose.

Curiosamente, foi possível observar a formação de vacúolos citoplasmáticos após exposição das células neuronais às β-ceto-anfetaminas por microscopia de contraste de fase, sugerindo a indução de autofagia. Os dados obtidos no último trabalho desta tese demonstraram que tanto a metilona como a MDPV induzem alterações morfológicas congruentes com ativação autofágica em neurónios dopaminérgicos, com formação de autofagossomas e ativação de marcadores de autofagia, tais como a formação de organelos vesiculares acídicos e aumento da expressão da proteína LC3-II, de uma forma dependente da concentração e do tempo de exposição. Os resultados mostraram ainda que a autofagia surge como um efeito primário da neurodegeneração induzida pelas β-ceto-anfetaminas, verificando-se a ativação dos marcadores autofágicos antes da indução de morte celular ou da ativação de caspase 3. Foi também demonstrado que o agente antioxidante *N*-acetil-L-cisteína detém efeitos neuroprotectores, levando a uma reversão completa da formação de espécies reativas de oxigénio e azoto e uma diminuição parcial de atividade autofágica e apoptótica induzidas pela metilona e pela MDPV, evidenciando a importância do stress oxidativo na regulação dos mecanismos de morte celular programada incitados pelas drogas de abuso em estudo.

Em conclusão, os resultados apresentados nesta tese contribuíram significativamente para a compreensão dos mecanismos inerentes aos efeitos hepatotóxicos e neurotóxicos das β-ceto-anfetaminas. Verificou-se que a toxicidade *in vitro* das catinonas sintéticas está relacionada com a indução de stress oxidativo e disfunção mitocondrial em ambos os órgãos-alvo, bem como a iniciação de morte celular por apoptose. Foi igualmente demonstrada a importância da autofagia nos efeitos neurodegenerativos destes psicostimulantes, com uma possível correlação entre stress oxidativo, autofagia e apoptose celular.

Palavras-chave: β-ceto-anfetaminas; hepatotoxicidade; neurotoxicidade; estudos *in vitro*.

Abstract

Synthetic cathinones, often termed β-keto amphetamines, are psychostimulant substances that have been emerging in the 21st century and flooded recreational settings as alternatives to illicit drugs of abuse, including amphetamines and cocaine. Inevitably, several cases of intoxications and deaths related to the abuse of β-keto amphetamines have been reported over the years. Adverse effects include severe agitation, hypertension, tachycardia, hyperthermia, paranoia and acute psychosis, as well as end-organ effects targeting the brain, liver, kidney, heart and muscle tissue. However, the mechanisms underlying target-organ toxicity have hitherto not been fully elucidated. Due to the chemical and pharmacological similarities shared with non-keto amphetamines, we hypothesized comparable toxicological mechanisms for cathinones.

The first purpose of the present work was to assess the *in vitro* hepatotoxic potential and the mechanisms of cytotoxicity elicited by four commonly abused cathinone derivatives, namely 3,4-methylenedioxymethcathinone (methylone or βk-MDMA), 3,4-methylenedioxypyrovalerone (MDPV), pentedrone and 4-methylethcathinone (4-MEC), using primary cultured rat hepatocytes (PRH) and differentiated HepaRG cells, in a comparative study with the well-known hepatotoxicant 3,4-methylenedioxymethamphetamine (MDMA).

Data obtained in this work showed that all β-keto amphetamines elicit hepatic cell death in both *in vitro* models in a concentration-dependent manner. Results from mechanistic studies support the ability of β-keto amphetamines to elicit *in vitro* hepatotoxicity through oxidative stress, mitochondrial dysfunction and apoptotic cell death. MDPV and pentedrone were the stronger hepatotoxic substances, even more potent than MDMA, whereas methylone was the least deadly derivative. The differences in drug potency herein found were directly correlated with its predicted lipophilicity. A partial reversion of cell death occurred in the presence of the CYP2D6 inhibitor, quinidine, supporting an important role for metabolic bioactivation in the overall hepatotoxic effects of synthetic cathinones.

Hyperthermia is a common clinical complication following β-keto amphetamines abuse, and the rise in temperature was determined to greatly potentiate the hepatotoxicity of amphetamines. Our data showed that the rise in temperature *per se* disturbed the redox system, and exacerbated all MDPV-elicited hepatotoxic effects, with enhancement in the production of reactive oxygen (ROS) and nitrogen (RNS) species, glutathione (GSH) and ATP depletion, and increased intracellular free calcium levels. Cell death by apoptosis was hastened under hyperthermic conditions, apparently shifting to necrosis at higher concentrations of MDPV.

The neurotoxic potential of the β-keto amphetamines methylone and MDPV was also studied *in vitro*, using dopaminergic SH-SY5Y cells. These drugs elicited concentration-dependent neuronal cell death, prompting the generation of ROS and RNS followed by the depletion of GSH, as well as mitochondrial impairment, characterized by the disruption of the mitochondrial membrane potential (Δψm) and ATP depletion. Apoptosis was also a common cell death pathway for methylone and MDPV, with activation of both intrinsic and extrinsic pathways, evident formation of pyknotic nuclei and chromatin condensation, and prevalence of cells in a late apoptotic stage. Interestingly, the formation of cytoplasmic vacuoles following exposure of neuronal cells to β-keto amphetamines was apparent under a phase-contrast microscopy, suggesting the induction of autophagy. The results of the last work showed that both methylone and MDPV instigated morphological changes consistent with autophagy in dopaminergic SH-SY5Y cells, including the formation of autophagosomes, and activation of autophagy markers, such as the formation of acidic vesicular organelles (AVOs) and increased expression of the autophagy-related protein LC3-II, in a time- and concentration-dependent manner. Importantly, data supported the triggering of autophagy as an early event of β-keto amphetamines-induced neurodegeneration, with the autophagic markers being activated prior to any significant effects on cell death or caspase 3 activation. Additionally, we showed that the antioxidant agent *N*-acetyl-L-cysteine (NAC) exhibits neuroprotective effects against methylone- and MDPV-induced cell death, leading to a complete inhibition of ROS and RNS generation, and partially deterring both apoptotic and autophagic activity, which supports a key role for oxidative stress in the regulation of programmed cell death pathways elicited by these drugs.

In conclusion, the data herein presented greatly contribute for a better understanding of the mechanisms underlying the hepatotoxic and neurotoxic effects of β-keto amphetamines, a prominent novel group of hazardous drugs of abuse. We showed that the *in vitro* cytotoxic effects of synthetic cathinones involve the triggering of oxidative stress and mitochondrial dysfunction in both target-organs. These substances also triggered intrinsic and extrinsic apoptotic cell death pathways in both neuronal and hepatic models. Autophagy was also shown to play a key role in the neurodegenerative effects of these psychostimulant drugs, with an apparent, but hitherto not fully understood, crosstalk between oxidative stress, autophagy and apoptotic cell death.

Keywords: β-keto amphetamines; hepatotoxicity, neurotoxicity; *in vitro* studies.

Thesis layout

This thesis is organized in 4 major sections.

Section I is a general introduction that comprises the current state of the art of β-keto amphetamine (cathinone) derivatives and their abuse. This section includes a review article for theoretical support entitled ***'Khat and synthetic cathinones: a review'*** (Valente MJ, Guedes de Pinho P, Bastos ML, Carvalho F, Carvalho M (2014) *Arch of Toxicol*, 88(1):15-45, doi: 10.1007/s00204-013-1163-9), and a brief review on the mechanisms of hepatotoxicity and neurotoxicity of β-keto amphetamines.

Section II encompasses the main objectives underlying this thesis, enacting the subsequent experimental wort presented.

Section III includes the main studies conducted for the purposes of this thesis, organized in four manuscripts of original research, including three published articles and one submitted manuscript.

Section IV presents an integrated discussion of all the work described in Section III.

Section V contains the main conclusions attained from the conducted work, as well as potential future studies.

Table of contents

List of abbreviations

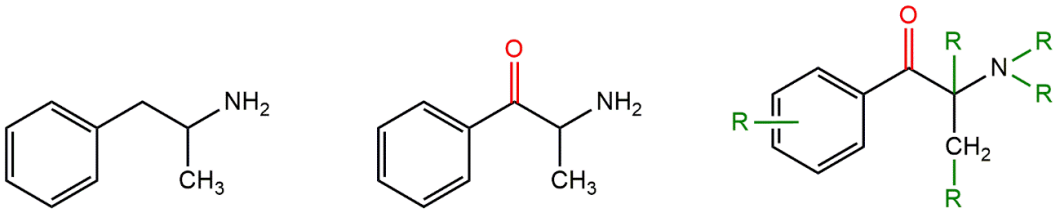
4-MEC	4-Methylethcathinone
4-MMC	4-Methylmethcathinone, mephedrone
5-HT	Serotonin, 5-hydroxytryptamine
α-MeDA	α-Methyldopamine
α-PVP	α-Pyrrolidinovalerophenone
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
CNS	Central nervous system
COMT	Catechol *O*-methyltransferase
CYP450	Cytochrome P450
Δψm	Mitochondrial membrane potential
DA	Dopamine
DAT	Dopamine transporter
DOPAL	3,4-Dihydroxyphenylacetaldehyde
DOPEGAL	3,4-Dihydroxyphenylglycolaldehyde
GFAP	Glial fibrillary acid protein
GSH	Glutathione (reduced form)
GSSG	Glutathione disulfide
IC_{50}	Half maximal inhibitory concentration
MAO	Monoamine oxidase
MDA	3,4-Methylenedioxyamphetamine
MDMA	3,4-Methylenedioxymethamphetamine
MDPV	3,4-Methylenedioxypyrovalerone

METH	Methamphetamine
N-Me-α-MeDA	*N*-Methyl-α-methyldopamine
NA	Noradrenaline
NAT	Noradrenaline transporter
NPS	New psychoactive substances
PRH	Primary rat hepatocytes
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SERT	Serotonin transporter
TEM	Transmission electron microscopy
TH	Tyrosine hydroxylase
VMAT	Vesicular monoamine transporter

Section I – General introduction

The global scenario of drug abuse has suffered a baffling transformation in the past few decades, with a shift of abuse patterns from classical illicit drugs to new psychoactive substances (NPS). NPS comprise hundreds of synthetic compounds from various chemical groups, which emerged as an attempt to outwit drug legislation through the manufacturing of psychostimulant drugs that were not controlled, ending up being labelled as 'legal highs' (Evans-Brown and Sedefov 2017). From 2009 to 2016, the Early Warning System of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) identified and currently monitors almost 600 NPS. From these, 103 are synthetic cathinones – about half detected between 2014 and 2015 –, making this the second largest group of NPS after synthetic cannabinoids (EMCDDA-Europol 2016; EMCDDA 2016).

Synthetic cathinones are derivatives of cathinone, a natural occurring psychostimulant alkaloid present in the fresh leaves of the *Catha edulis* (or khat) plant, structurally similar to amphetamine (figure 1). They began to be sold legally online or at *smartshops* as alternatives to illicit drugs such as 3,4-methylenedioxymethamphetamine (MDMA) and cocaine, labelled as 'bath salts' and 'plant feeders' (figure 2) and deceptively advertised as 'not for human consumption' (Valente et al. 2014). Due to their chemical resemblance to amphetamine derivatives, with the difference being the presence of a ketone group in the β-position of the alkyl side chain (figure 1), these designer drugs are often termed β-keto amphetamines (Zaitsu et al. 2011).



Amphetamine | **Cathinone** (β-keto amphetamine) | **Cathinone derivatives** (β-keto amphetamines)

Figure 1: Chemical structures of amphetamine and cathinone, and general structural representation of cathinone derivatives.

Notwithstanding all the efforts made so far to control the manufacturing and marketing of synthetic cathinones (Valente et al. 2014), dozens of new structural derivatives emerge in the recreational markets every year, hindering the development of an effective

regulatory system for these NPS. Furthermore, several derivatives that are already under legal control conquered a prominent place in illicit drug markets. This is the case of 4-methylmethcathinone (4-MMC or mephedrone), 3,4-methylenedioxymethcathinone (methylone or βk-MDMA) and 3,4-methylenedioxypyrovalerone (MDPV), which were the first three synthetic cathinones to be scheduled as controlled substances in the United States and in several European Union member states, and are still three of the most commonly abused derivatives (Ashrafioun et al. 2016; Bretteville-Jensen et al. 2013; EMCDDA-Europol 2016; EMCDDA 2016; van Amsterdam et al. 2015), being responsible for several cases of intoxication and deaths even after banning (Busardo et al. 2015; Grapp et al. 2017; Karila et al. 2016).

Figure 2: Packages of 'plant feeders' containing synthetic cathinones. *Material apprehended by the Portuguese Judicial Police after the closing of local smartshops due to the new legislative control measures regarding 'legal highs' in force since April 2013, which penalizes the commercialization and use of a total of 159 NPS, including 33 cathinone derivatives.*

Depending on the structural substitution(s) made to the original molecule of cathinone, synthetic cathinones are divided into different chemical groups, and the pharmacological and toxicological properties, as well as the subjective and adverse effects may vary among groups and derivatives (Valente et al. 2014).

Subjective and adverse effects of synthetic cathinones range from amphetamine- to cocaine-like, depending on their chemical properties and pharmacological mechanisms. General desired effects include euphoria, enhanced sociability and empathy feelings, creativity enhancement, increased state of wakefulness and alertness, and increased libido. The most common adverse clinical symptoms are cardiac, neurological and psychiatric effects, consistent with the sympathomimetic toxidrome, and characterized by severe hypertension, tachycardia, chest pains, seizures, aggressiveness and extreme agitation, hyperthermia, paranoia, delirium and acute psychosis (Hohmann et al. 2014; Prosser and Nelson 2012; Spiller et al. 2011; Valente et al. 2014). Abuse of cathinones also induces end-organ effects, including rhabdomyolysis, cerebral and lung edema, myocardial infarction, acute renal and liver failure, or even multiorgan system failure, which may ultimately lead to death (Adebamiro and Perazella 2012; Borek and Holstege 2012; Hohmann et al. 2014; Omer and Doherty 2011; Ross et al. 2012; Valsalan et al. 2017; Warrick et al. 2012).

The general introduction of this thesis intends to provide an overview of the current state of the art of β-keto amphetamine (cathinone) derivatives and their abuse. It includes a review article (‘Khat and synthetic cathinones: a review’), providing detailed information on historical background, prevalence and patterns of abuse, legal status, chemical aspects, pharmacokinetics and pharmacodynamics, subjective and adverse effects of these psychoactive substances. It also includes a brief review on the mechanisms of hepatotoxicity and neurotoxicity of β-keto amphetamines.

Review article. Khat and synthetic cathinones: a review

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REVIEW ARTICLE

Khat and synthetic cathinones: a review

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Abstract For centuries, 'khat sessions' have played a key role in the social and cultural traditions among several communities around Saudi Arabia and most East African countries. The identification of cathinone as the main psychoactive compound of khat leaves, exhibiting amphetamine-like pharmacological properties, resulted in the synthesis of several derivatives structurally similar to this so-called natural amphetamine. Synthetic cathinones were primarily developed for therapeutic purposes, but promptly started being misused and extensively abused for their euphoric effects. In the mid-2000's, synthetic cathinones emerged in the recreational drug markets as legal alternatives ('legal highs') to amphetamine, 'ecstasy', or cocaine. Currently, they are sold as 'bath salts' or 'plant food', under ambiguous labels lacking information about their true contents. Cathinone derivatives are conveniently available online or at 'smartshops' and are much more affordable than the traditional illicit drugs. Despite the scarcity of scientific data on these 'legal highs', synthetic cathinones use became an increasingly popular practice worldwide. Additionally, criminalization of these derivatives is often useless since for each specific substance that gets legally controlled, one or more structurally modified analogs are introduced into the legal market. Chemically, these substances are structurally related to amphetamine. For this reason, cathinone derivatives share with this drug both central nervous system stimulating and sympathomimetic features. Reports of intoxication and deaths related to the use of 'bath salts' have been frequently described over the last years, and several attempts to apply a legislative control on synthetic cathinones have been made. However, further research on their pharmacological and toxicological properties is fully required in order to access the actual potential harm of synthetic cathinones to general public health. The present work provides a review on khat and synthetic cathinones, concerning their historical background, prevalence, patterns of use, legal status, chemistry, pharmacokinetics, pharmacodynamics, and their physiological and toxicological effects on animals and humans.

Keywords Khat · Synthetic cathinones · Bath salts · Pharmacokinetics · Pharmacodynamics · Toxicity

Introduction

Cathinone is the major naturally occurring psychoactive component found in the leaves of the *Catha edulis* plant, commonly known as khat. The first cathinone derivatives were originally synthesized in the beginning of the twentieth century for therapeutic purposes, but it was only in the last decade that the recreational use of these synthetic compounds gained public attention (Balint et al. 2009; Kelly 2011). Synthetic cathinones are included in a larger group of psychoactive substances generally designated by 'legal highs'. They are indiscriminately abused for their cocaine and amphetamine-like pharmacological effects, usually

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labeled 'not for human consumption', and sold as 'plant food' or 'bath salts' in order to bypass legislative restrictions in several countries (Bretteville-Jensen et al. 2013; Fass et al. 2012; Van Hout and Brennan 2011).

Methcathinone (ephedrone, 'CAT') and 4-methylmethcathinone (mephedrone, 4-MMC) were the first cathinone derivatives to be produced. Mephedrone, along with methylone (3,4-methylenedioxy-*N*-methylcathinone, βk-MDMA) and MDPV (3,4-methylenedioxypyrovalerone), rapidly emerged in recreational drug markets as the main ingredients of 'bath salts', becoming readily accessible on the Internet and at the so-called 'head' or 'smartshops'. By the end of 2011, these three compounds were provisionally scheduled in the United States of America (USA) under drug legislation for further analysis of potential harm (Jerry et al. 2012; Kelly 2011; Prosser and Nelson 2012). Nonetheless, legal regulation of synthetic drugs like cathinone derivatives is rather difficult to attain success, since they are easily replaced by novel compounds after minor structure modifications. Consequently, for each drug that gets banned, new and more powerful analogs will reach the licit drug markets.

Due to their legal status, users are deceivingly led to believe that these drugs are safe to consume. However, several cases of 'bath salts'-related intoxication and deaths have been reported over the last years (James et al. 2011; Kovacs et al. 2012; Murray et al. 2012; Wood et al. 2010a). Despite the scarcity of experimental data on the pharmacological and toxicological properties of these 'legal highs', based on the structural similarities of cathinone derivatives with other amphetamines like MDMA (3,4-methylenedioxymethamphetamine, 'ecstasy'), identical effects are predictable.

The purpose of this work is to provide a thorough report on the currently known synthetic cathinone derivatives, and to review their chemical, pharmacological, and toxicological properties.

Historical background

Khat (*Catha edulis*) is a flowering evergreen plant that grows wild in the Horn of Africa and in the Southwest Arabian Peninsula. For centuries, the chewing of fresh khat leaves, for their gratifying stimulant effects, has been a tradition in local communities, especially at cultural and religious ceremonies, including funerals and weddings. Khat chewing is also widely practiced on a daily basis, at the so-called khat sessions, where men gather and chat for several hours, usually after work. These sessions are a major social and cultural phenomenon in this area, particularly in Yemen (Al-Motarreb et al. 2002; Balint et al. 2009; Carvalho 2003).

Recently, and following the improvement of the routes for transportation and distribution, the availability of khat leaves ceased to be exclusive to the native regions, and immigrants spread their use to Western countries (Alem et al. 1999; Griffiths et al. 2010), although with the limitation that only fresh khat leaves may achieve the aimed psychotropic effects.

The khat plant was first described during an expedition to Egypt and Yemen in 1761–1763, by a Swedish botanist named Peter Forskal, who identified *C. edulis* as a member of the family Celastraceae. In 1775, Karsten Niebuhr, Forskal's travelling companion and the only survivor of the expedition, named khat as *C. edulis* Forsk (Al-Motarreb et al. 2002; Dhaifalah and Santavy 2004; Kalix 1984).

The fresh khat leaves contain over forty compounds, including alkaloids, tannins, flavonoids, terpenoids, sterols, glycosides, amino acids, vitamins, and minerals (Balint et al. 2009; Cox and Rampes 2003; Halbach 1972; Kalix 1984). In the first attempt to identify the active principle(s) of khat, Fluckiger and Gerock (1887) detected a psychoactive compound they named *katin*, which was later identified by Wolfes (1930) as (+)-norpseudoephedrine, an *Ephedra* alkaloid. For the following three decades, (+)-norpseudoephedrine, commonly known as cathine, was believed to be the major active component of khat, although some evidences showed that cathine stimulant effect was insufficient as the sole responsible for khat pharmacological effects (Halbach 1972; Kalix 1984; Szendrei 1980; Zelger et al. 1980). Thus, in 1975, a βk-analog and precursor of cathine, the (−)-α-aminopropiophenone, or cathinone, was isolated in the United Nations' Narcotics Laboratory (United Nations 1975). Early studies showed that cathinone is 7- to 10-fold more potent than cathine but degrades rapidly, thus explaining the need to chew fresh khat leaves (Cox and Rampes 2003; Kalix and Khan 1984; Kelly 2011; Knoll 1979; Nencini and Ahmed 1989). Cathinone appeared in the recreational Israeli markets in the early 2000's, in 200 mg capsules, under the brand name 'Hagigat', which was sold as a natural psychostimulant and aphrodisiac (Bentur et al. 2008).

Besides cathine, cathinone can also be metabolized into (−)-norephedrine (Brenneisen et al. 1986). These three alkaloids belong to the khat phenylalkylamine family.

With the purpose of synthesizing a series of ephedrine homologs, Hyde et al. (1928) optimized earlier methods and obtained purified α-methylaminopropiophenone, also known as methcathinone or ephedrone (EPH). In the following year, Saem de Burnaga Sanchez (1929) described a method for the chemical synthesis of 4-methylmethcathinone, generally named mephedrone (MEPH).

Due to their central nervous system (CNS) stimulant properties, some synthetic cathinone derivatives, such as *m*-chloro-*N*-*tert*-butyl-cathinone (bupropion),

N,N-diethylcathinone (diethylpropion, amfepramone), and *N,N*-dimethylcathinone (dimethylpropion, metamfepramone), were primarily developed for therapeutic purposes, mainly as antidepressants and anorectic drugs (Canning et al. 1979; Cunningham 1963; Markantonis et al. 1986; Seaton et al. 1961; Soroko et al. 1977). However, whereas these three derivatives exhibited minor misuse potential, EPH, which was also meant to be marketed as an antidepressant, displayed strong addictive potential, with a cocaine-like stimulus more potent than cocaine and cathinone itself (Young and Glennon 1993). This resulted in EPH misuse and abuse, known by the street names 'Jeff' or 'Cat', first in the former Soviet Union and later in the USA (Clein and Benady 1962; Emerson and Cisek 1993; Goldstone 1993; Griffith et al. 1983; Kuenssberg 1962). Consequently, since the early 90's, EPH has been implicated in several cases of intoxications. It can be easily synthesized at home through the oxidation of readily available pharmaceuticals containing ephedrine or pseudoephedrine with potassium permanganate, in the presence of acetic acid. As a result, EPH intoxications typically manifest as a manganese-induced Parkinsonism (Belhadj-Tahar and Sadeg 2005; Colosimo and Guidi 2009; de Bie et al. 2007; Gorgaslidze et al. 1993; Iqbal et al. 2012; Sanotsky et al. 2007; Varlibas et al. 2009).

A few years later, in 1996, methylone was synthesized and patented as an antidepressant and anti-Parkinsonism agent. With a psychostimulant potency close to the one observed with MDMA itself, methylone ended not being ever marketed for the expected purposes (Dal Cason et al. 1997).

By the same time, derivatives of α-PPP (α-pyrrolidino propiophenone), the pyrrolidine *N*-substituted cathinone, started appearing during seizures on the German drug market (Maurer et al. 2004; Springer et al. 2003c; Westphal et al. 2007). Similarly to other aminopropiophenone derivatives, several substances from pyrrolinophenone family were initially synthesized for clinical uses. In the early 1970's, 4-methyl-α-pyrrolidinopentanophenone, or pyrovalerone, was developed to treat obesity, chronic fatigue, and lethargy (Gardos and Cole 1971; Goldberg et al. 1973; Prosser and Nelson 2012). However, due its strong addictive properties, and after the report of abuse by polydrug users, pyrovalerone was withdrawn from clinical treatments (Deniker et al. 1975; Prosser and Nelson 2012; Sauer et al. 2009; Yohannan and Bozenko Jr 2010). Later, some α-PPP derivatives with a potent central muscle relaxant effect entered the Japanese markets (Morikawa et al. 1987; Sakitama et al. 1995; Yamazaki et al. 1987).

The pyrrolidinophenone family includes several other psychoactive substances that, unlike pyrovalerone, were never intended for therapeutic use. These substances are MPPP (4-methyl-α-pyrrolidinopropiophenone), MPHP (4-methyl-α-pyrrolidinohexiophenone), MPBP (4-methyl-α-pyrrolidinobutiophenone), MOPPP (4-methoxy-α-pyrrolidinopropiophenone), MDPPP (3,4-methylenedioxy-α-pyrrolidinopropiophenone), and MDPV (Peters et al. 2005; Springer et al. 2002, 2003a, b, d).

Recreationally, the first generation of synthetic cathinones to be sold in the markets includes methylone, which emerged in the mid-2000's under the brand name 'Explosion', first in the Netherlands and Japan, and later in Australia and New Zealand. This was the first of these substances to be marketed via the Internet and at smartshops, making it readily accessible, affordable, and easy to purchase (Bossong et al. 2005; Maheux et al. 2010; Zaitsu et al. 2011). Since then, methylone poisonings have been frequently reported, in single consumption (Carbone et al. 2013; Kovacs et al. 2012), but mostly in combination/mixture with other psychoactive substances (Boulanger-Gobeil et al. 2012; Cawrse et al. 2012; Pearson et al. 2012; Shimizu et al. 2007; Warrick et al. 2012).

MEPH, colloquially known as 'M-Cat', 'Meph', 'Subcoca', 'TopCat', or 'miaow miaow', is another first-generation cathinone derivative. It is originally from Israel, where it started being sold as a legal alternative to cocaine or 'ecstasy', and available in capsules named Neodove at the online company Neorganics (Brunt et al. 2011; Deluca et al. 2009b; Vekariya 2012).

Possibly the most well-known and more studied substance in the market of synthetic cathinones, MEPH became popular in Europe only recently, right after it was banned in Israel, by 2008. MEPH was first identified in Finland, in March 2008, and was reported in the same year to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) and Europol, via the European Union (EU) early-warning system (EMCDDA-Europol 2009; Kelly 2011). This interest in MEPH was promptly disseminated throughout all Europe, especially in the United Kingdom (UK) and Ireland, and even Australia (Bruno et al. 2012; James et al. 2011; McElrath and O'Neill 2011; Measham et al. 2010; Van Hout and Brennan 2011). However, it was only in 2010 that the first case of MEPH exposure in the USA was reported by the National Poison Data System (Bronstein et al. 2011).

Besides MEPH, other five synthetic cathinone derivatives were identified and reported in 2008, namely ethcathinone (*N*-ethylcathinone, ethylpropion), flephedrone (4-fluoromethcathinone, 4-FMC) and its positional isomer 3-fluoromethcathinone (3-FMC), butylone (β-keto-methylbenzodioxolylbutanamine, βk-MBDB), and MDPV (EMCDDA-Europol 2009).

Evidences on ethcathinone recreational use are scarce, and, aside one case of poisoning with ethcathinone combined with methylone reported last year (Boulanger-Gobeil et al. 2012), little is known about this substance.

Equally lacking information in the literature are buphedrone (α-methylaminobutyrophenone, MABP) and methedrone (4-methoxymethcathinone). Buphedrone was one of the series of ephedrine homologs synthesized in 1928 by Hyde et al. (1928) and, as a positional isomer of MEPH, like ethcathinone, was one of the first choices for alternative 'legal high' after MEPH was banned. Two cases of buphedrone use were reported in Poland, one of which was a fatal case as a result from a car crash for driving under the influence of this substance and MDPV (Zuba et al. 2013).

Like buphedrone, methedrone emerged as a MEPH substitute. Two fatal cases related with methedrone intake were reported in Sweden by 2009, which contributed for the decision of the Swedish government to schedule this substance as a narcotic drug by the end of that year (Wikstrom et al. 2010).

The fluorinated MEPH derivatives, flephedrone and 3-fluoromethcathinone, were the next derivatives to reach the markets, followed by butylone and ethylone (3,4-methylenedioxy-*N*-ethylcathinone, bk-MDEA), and finally MDPV (Archer 2009; EMCDDA-Europol 2009; Zaitsu et al. 2009). Contrary to ethcathinone, these five cathinone derivatives, along with methylone, became commonly available for purchase, usually in the mixtures of several cathinones and other psychoactive drugs (Zuba and Byrska 2013). Public interest on these derivatives, as alternatives to MEPH, greatly increased after the last one became controlled in the EU. Nonetheless, MEPH remained available in the illicit drug market for the following period and is still currently a matter of concern to public health (EMCDDA 2012; McElrath and O'Neill 2011). Attesting this tendency, EMCDDA-Europol notified, in the annual joint implementation report, a record number of 15 new synthetic cathinones being marketed in 2010, versus the 4 derivatives reported in 2009 and 6 in the year before (EMCDDA-Europol 2009, 2010, 2011).

Due to the continuous search for new, legal, less expensive, and more powerful highs by drug users, the synthesis of novel cathinone derivatives became a fruitful industry, leading to a fast emergence of new alternative substances every year. As a consequence of the criminalization of MEPH derivatives, a group of products named 'Energy' (NRG), advertised as naphthyl cathinone analogs, started entering the recreational scene (Measham et al. 2011). NRG-1, the first one of these preparations to be marketed, was purported to contain naphthylpyrovalerone, or naphyrone, which was also the first of the second-generation synthetic derivatives to be reported via the EU early-warning system, in 2010 (EMCDDA-Europol 2011). Nonetheless, studies on the chemical composition of NRG-1 revealed that only a minor part of the available preparations of this brand actually contained naphyrone. In fact, Brandt et al. (2010a, b, 2011) showed that most samples of NRG-type products analyzed contained illegal synthetic cathinones, namely MEPH, flephedrone, butylone, MDPV, pentylone (β-keto-methylbenzodioxolylpentanamine, βk-MBDP), 4-MEC (4-methyl-*N*-ethylcathinone), MDPBP (3,4-methylenedioxy-α-pyrrolidinobutiophenone), and MPPP, which raises serious health concerns to users and demands criminal repercussions to sellers.

By the same time, the so-called third-generation synthetic cathinones started showing up, first 3,4-DMMC (3,4-dimethylmethcathinone) and then pentedrone (α-methylaminovalerophenone) and α-PVP (α-pyrrolidinovalerophenone; α-pyrrolidinopentiophenone) (EMCDDA-Europol 2010, 2011). Aside a few studies on their chemical characterization, metabolism, and distribution (Locos and Reynolds 2012; Maheux and Copeland 2012; Marinetti and Antonides 2013; Shima et al. 2013), there is limited toxicological data in the literature regarding these substances and for most of the derivatives reported so far.

Figure 1 summarizes the major historical events associated with khat and synthetic cathinone derivatives.

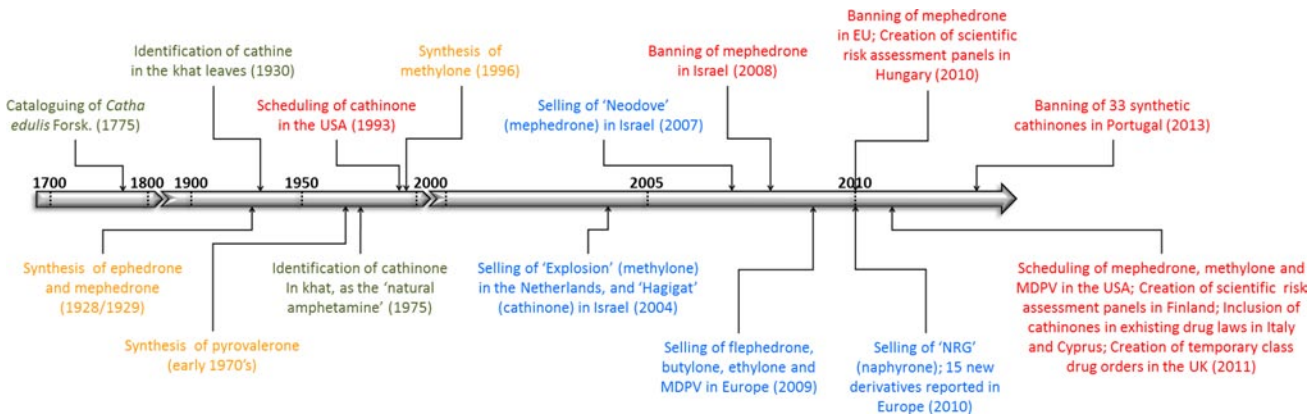

Fig. 1 Time line of the main events related to the khat plant and synthetic cathinones

Prevalence, patterns of use, and legal status

The majority of khat chewers is located in Yemen, where recent reports estimate that up to 90 % of the adult males and approximately 50 % of the general adult population consume khat leaves on a daily basis. High prevalence is also observed in many East African countries, including Somalia, Ethiopia, Uganda, and Kenya (Al-Motarreb et al. 2002; Al-Mugahed 2008; Alem et al. 1999; Dhaifalah and Santavy 2004; Manghi et al. 2009; Tesfaye et al. 2008).

The use of khat was, for many decades, confined to the regions where *C. edulis* grows. However, due to the improvement of distribution routes, the emergence of online markets for fresh khat leaves, and the immigration from native countries to Europe and USA, khat chewing has been globalized (Alem et al. 1999; Cox and Rampes 2003; Feyissa and Kelly 2008; Griffiths et al. 2010).

Khat leaves are identified by many local names, including qat, qaat, chat, and miraa. They are harvested early in the morning and sold in bundles of about 100–200 g of fresh leaves wrapped in banana leaves in order to preserve their freshness. Chewing one bundle per day is considered a moderate and usual consumption. There are several types of khat bundles available, and prices vary according to the quality and potency of their effects. In Eastern Africa countries, khat may cost from US0.5$ for the cheaper and less potent type, and up to US20$ per kilo of cleaner and stronger leaves. In the United Kingdom, each bundle costs between 3 and 7£ (US4.7 to 11$), but it may reach the 50$ in the USA (Balint et al. 2009; Cox and Rampes 2003; Fasanmade et al. 2007; Hansen 2010; Klein et al. 2009; Klein et al. 2012; Patel et al. 2005).

Currently, khat legal status is ambiguous. In 1993, cathinone was placed into Schedule I of the Controlled Substances Act, whereas cathine was already ruled as a Schedule IV substance in 1988 (Drug Enforcement Administration 1993a, b). Khat may fall into Schedule I substances when containing detectable levels of cathinone. Nonetheless, its legal status is often challenged, especially among immigrants living in countries where khat is legally controlled (Klein et al. 2012). Khat is currently illegal in the USA and Canada, and was banned in several European countries, including Ireland, France, Denmark, Germany, Sweden, Norway, and more recently the Netherlands. However, it is still legal in the United Kingdom, Portugal, Australia, and in most Eastern Africa countries (Arunotayanun and Gibbons 2012; Gezon 2012; Klein et al. 2012).

All over the world, the development of new drugs has been a target of increasing public attention, mostly in the last decade. In Europe, over 150 novel psychoactive substances were reported to the EMCDDA from 2005 to 2011, from which 34 were synthetic cathinones (EMCDDA-Europol 2011). Cathinone analogs, together with synthetic cannabinoids, represent two-thirds of all the notified substances since 2005, in a group that includes also legal phenethylamines, piperazines, and tryptamines, the so-called legal highs (EMCDDA 2012).

In order to circumvent legislative control, synthetic cathinones are ambiguously advertised as 'bath salts', 'plant food', or even 'hoover freshener', and labeled 'not for human consumption' (Bretteville-Jensen et al. 2013; Fass et al. 2012; Van Hout and Brennan 2011).

'Bath salts' are sold under several inexplicit brand names, including Bloom, Blue Silk, Ivory Wave, Purple Wave, and Vanilla Sky, and purchased locally at convenience stores and head- or smartshops, or conveniently over Internet suppliers, being readily accessible, affordable, and technically legal (Coppola and Mondola 2012; Gershman and Fass 2012; Kelly 2011; Mas-Morey et al. 2012; Spiller et al. 2011; Volkow 2011). Nonetheless, there is a lack of information, reliability, and consistency on the chemical composition of the available products, and most of those allegedly containing legal substances are actually composed by formerly banned compounds, the most common being MEPH, methylone, and MDPV (Brandt et al. 2010b; Fass et al. 2012; Prosser and Nelson 2012; Ramsey et al. 2010). As a result, despite their recent scheduling as controlled substances within the USA and elsewhere, illegal synthetic cathinones frequently appear in psychoactive products labeled as 'bath salts' still on the market. Additionally, as the law is always a step behind, several new cathinone derivatives keep emerging in the recreational markets every year to avoid detection or legal scrutiny.

Synthetic cathinones are generally sold in the form of a white or yellowish amorphous or crystalline powder, or in capsules, and are usually found as 200 mg to 10 g packets, costing approximately 10–20$ per gram, but can also be purchased in bulk quantities for discounts. Cathinone derivatives in tablets are more uncommon (Bretteville-Jensen et al. 2013; Coppola and Mondola 2012; Dargan et al. 2011; Fass et al. 2012; Karila and Reynaud 2011; Kelly 2011; Prosser and Nelson 2012; Yohannan and Bozenko Jr 2010).

MEPH is usually found as a white or colored hydrochloride salt and is likely to be sold as a racemic mixture of the stereoisomeric *R* and *S* forms. An unpleasant odor to the compound, as a mixture of chloride, vanilla, and urine, was described by MEPH users. MDPV, commonly available as a white light tan powder, was also reported to develop an odor when exposed to air (Gibbons and Zloh 2010; Gorun et al. 2010; Yohannan and Bozenko Jr 2010).

Ingestion, either by swallowing capsules or 'bombing' (the powder is swallowed after being wrapped in a cigarette paper) (Deluca et al. 2009b), and nasal insufflation ('snorting'), more specifically by 'keying' (a key is dipped in the powder and then insufflated) (Lindsay and White 2012) are

the main routes for the administration of these substances. Inhalation, gingival and sublingual delivery, intravenous injection, and rectal administration have also been reported (Coppola and Mondola 2012; Gershman and Fass 2012; Karila and Reynaud 2011; Lindsay and White 2012; Mas-Morey et al. 2012; Prosser and Nelson 2012).

The increasing popularity of these substances may be linked to several reasons. Users resort to the synthetic cathinones for their cocaine and MDMA-like psychostimulant effects, but are usually misled by the term 'legal high', and thus often believe they are safe to consume. Nevertheless, the fact that they can be legally purchased may be persuasive for both new consumers and illicit drug users. Furthermore, it was also suggested a connection between the peak of synthetic cathinones demand in 2010 and the loss of purity of cocaine samples and 'ecstasy' pills seized in 2009 (Measham et al. 2010).

Estimations on the prevalence of synthetic cathinones are very difficult to attain, and surveys on self-report abuse are still currently the only source for this matter. However, the majority of these surveys corresponds to specific groups and, consequently, is not representative of the general population.

An online survey targeting UK club-goers conducted by the end of 2009 reported that 41.3 % of the 2,295 respondents had used MEPH, with a total of 15.1 % reporting a weekly or more frequent use. On the other hand, only 10.8 and 1.9 % had consumed methylone and MDPV, respectively (Winstock et al. 2011). MEPH was determined to be the fourth more commonly used drug in Europe, after cannabis, 'ecstasy', and cocaine, sixth if considering alcohol and tobacco, and the most abused 'legal high' (Europol–EMCDDA 2010; Winstock et al. 2011).

The UK club scene is one of the most thriving markets for MEPH abuse, even after its legal restriction. In fact, in an in situ drug survey conducted at two 'gay-friendly' South London dance clubs by 2011, 41 % of the respondents had taken or planned to take MEPH on the night of the survey, against the 27 % determined in 2010 in the same conditions (Measham et al. 2011). Of note, MEPH was considered the favorite drug of all (20.4 %), followed by cocaine (14.9 %) (Wood et al. 2012).

Also in the United Kingdom, a self-report questionnaire conducted in high schools, colleges, and universities in Scotland revealed that 20.3 % of the students had used MEPH at least once, 4.4 % consumed it on a daily basis, and 17.6 % had already experienced addiction or dependence symptoms (Dargan et al. 2010).

The 2010/2011 British Crime Survey found that the prevalence of MEPH use among adults aged 16–59 was roughly the same as the one observed with 'ecstasy' (1.4 %), whereas among users from 16 to 24 years, the levels of use of this synthetic cathinone were comparable to powder cocaine (both 4.4 %) (Smith and Flatley 2011).

The concomitant use of synthetic cathinones with other substances is a common practice worldwide, and it may include other substituted cathinones, several kinds of 'legal highs', illicit and prescription drugs, anesthetic agents, and alcohol (Deluca et al. 2009b; James et al. 2011; Karila and Reynaud 2011; Maskell et al. 2011).

In Finland, the prevalence of MDPV use was assessed in drivers suspected to be under the influence of drugs. From August 2009 to August 2010, 5.7 % (259 drivers) of all cases of confirmed driving under the influence of drugs were found positive for MDPV, with blood concentrations ranging from 0.016 to 8.400 mg/l. Alcohol was present in a minor part of these cases (only 22 of the 259 drivers), and regularly under the legal limit for alcohol in Finland. However, 80 and 67 % of the MDPV-positive drivers were also positive for amphetamine and benzodiazepines, respectively, and 54 % had the 3 types of drugs combined (Kriikku et al. 2011).

In Ireland, 13.9 % of a total number of 209 urine samples from attendees at the Drug Treatment Centre Board were positive for MEPH, while methylone was found in 3.3 % of the samples (McNamara et al. 2010).

An Australian study showed that the consumption of cathinone derivatives is frequent among regular 'ecstasy' users (17 and 0.5 % using MEPH and MDPV, respectively) (Bruno et al. 2012).

Also, in Australia, an online prevalence assessment targeting same-sex-attracted people between 18 and 25 years old revealed that 4 % of the respondents had been using MEPH (Lea et al. 2011), which is proportionally lower than the UK findings, suggesting that synthetic cathinones may have entered the European markets in a much higher degree than in Australia.

Statistics on the prevalence of use of synthetic cathinones in the USA are limited. Recent data from the American Association of Poison Control Centers (AAPCC) reported a significant increase in calls related to 'bath salts' exposure from 2010 (304) to 2011, with a record number of 6136 calls, followed by a decrease to 2,656 calls in 2012 (AAPCC 2013). When comparing the number of calls made to national poison control centers in the USA and the United Kingdom, between 2009 and 2012, it was observed a similar number normalized per month and population. However, the peak of calls in the United Kingdom occurred more than one year earlier than in the USA (Spyker et al. 2012).

The legal status of synthetic cathinones differs greatly from country to country, and even between states, and is always changing according to new findings on possible risks for the public safety.

After the report of several cases of intoxication and some MEPH-related deaths in Europe (Maskell et al. 2011), EMCDDA conducted a risk assessment report on this substance (EMCDDA 2011). Supported by the report findings, MEPH was classified as a controlled drug under the UK Misuse of Drugs Act of 1971 and was banned, along with other cathinone derivatives, on April 2010 (Morris 2010; Vardakou et al. 2012).

Europe has responded to the rising concern over the use of MEPH by subjecting it to 'control measures and criminal penalties' throughout the EU. In a Decision of 2 December, the Council of the EU banned the drug, calling on Member States to introduce controls in line with their national law (Council 2010).

In Portugal, new legislative control measures regarding 'legal highs' were introduced last April, penalizing the commercialization and use of a total of 159 substances, and 33 of them are synthetic cathinones. The Portuguese Government (2013) remarked as being illegal to produce, import, export, market, distribute, advertise, deliver, or possess these substances, and this ultimately resulted in the closing of smartshops all over the country. This new law also predicts a progressive update of the list of controlled substances every 18 months.

Until the fourth trimester of 2011, the synthetic cathinones were unscheduled in the USA, but human consumption was considered illegal under the Federal Analog Act of 1986. On October 21, 2011, the Drug Enforcement Administration temporary scheduled MEPH, methylone, and MDPV, under Schedule I of the Controlled Substances Act, criminalizing the sale and possession of these substances until final ruling on the requirement for further control. The scheduling of methylone was later extended until last April, and all three derivatives remained Schedule I substances and are permanently banned in the USA (Bretteville-Jensen et al. 2013; Drug Enforcement Administration 2011, 2012).

Chemistry

Synthetic cathinones are phenylalkylamine derivatives, closely related to amphetamines, the difference being the ketone group introduced at the β-position of the amino alkyl chain attached to the phenyl ring. For this reason, these 'legal highs' are often entitled βk-amphetamines (Zaitsu et al. 2011).

Cathinone derivatives are analogs of the natural cathinone (Fig. 2a) and are synthesized by adding diverse substituents at different locations of the cathinone molecule, as depicted in Fig. 2b.

So far, more than 30 cathinone derivatives have been identified in the licit and illicit recreational drug markets,

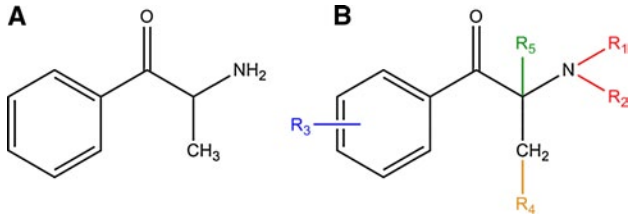

Fig. 2 Chemical structure of cathinone (**a**) and general structure of synthetic cathinones (**b**)

Table 1 Synthetic cathinones: common names and corresponding chemical designation

Common names	Chemical name
Ephedrone, methcathinone	α-Methylaminopropiophenone
Mephedrone, 4-MMC	4-Methylmethcathinone
Bupropion, amfebutamone	*m*-Chloro-*N*-*tert*-butyl-cathinone
Dimethylpropion, metamfepramone	*N,N*-Dimethylcathinone
Diethylpropion, amfepramone	*N,N*-Diethylcathinone
Flephedrone, 4-FMC	4-Fluoromethcathinone
3-FMC	3-Fluoromethcathinone
Ethcathinone, ethylpropion	*N*-Ethylcathinone
4-MEC	4-Methylethcathinone
Methedrone	4-Methoxymethcathinone
Buphedrone	α-Methylaminobutyrophenone
Pentedrone	α-Methylaminovalerophenone
3,4-DMMC	3,4-Dimethylmethcathinone
Methylone, βk-MDMA	3,4-Methylenedioxy-*N*-methylcathinone
Ethylone, βk-MDEA	3,4-Methylenedioxy-*N*-ethylcathinone
Butylone, βk-MBDB	β-Keto-methylbenzodioxolylbutanamine
Pentylone, βk-MBDP	β-Keto-methylbenzodioxolylpentanamine
α-PPP	α-Pyrrolidinopropiophenone
Pyrovalerone	4-Methyl-α-pyrrolidinopentanophenone
α-PVP	α-Pyrrolidinovalerophenone
MPPP	4-Methyl-α-pyrrolidinopropiophenone
MOPPP	4-Methoxy-α-pyrrolidinopropiophenone
MPBP	4-Methyl-α-pyrrolidinobutiophenone
MPHP	4-Methyl-α-pyrrolidinohexiophenone
MDPV	3,4-Methylenedioxypyrovalerone
MDPPP	3,4-Methylenedioxy-α-pyrrolidinopropiophenone
MDPBP	3,4-Methylenedioxy-α-pyrrolidinobutiophenone
Naphyrone	Naphthylpyrovalerone

with more or less data available in the literature for each substance. In Table 1 are resumed the main synthetic derivatives available so far.

Chemically, this group of 'legal highs' can be separated into four families. The first known synthetic cathinone analogs were frequently *N*-alkylated cathinones at R_1 and/or R_2, some of which with ring substituents (R_3). This family of cathinones includes substances that were primarily synthesized for therapeutic purposes, namely the anorectics diethylpropion and dimethylpropion and the antidepressant bupropion, and derivatives that were in fact introduced into the recreational drug market: ethcathinone, EPH, MEPH, flephedrone (and its isomer 3-FMC), 4-MEC, methedrone, buphedrone, pentedrone, and 3,4-DMMC (Fig. 3).

Instead of an alkylation or halogenation at R_3, a 3,4-methylenedioxy group can be added to the benzyl ring (Dal Cason 1997). This group encompasses the *N*-methylated and *N*-ethylated derivatives methylone and ethylone, and also butylone and pentylone, which result from an alkylation at R_1 and R_4, respectively (Fig. 4a). Of note, this family of cathinone derivatives is structurally similar to 3,4-methylenedioxyamphetamines (Fig. 4b), some of which regularly abused substances, namely MDMA, MDEA (3,4-methylenedioxyethamphetamine), and MBDB (3,4-methylenedioxy-α-ethyl-*N*-methylphenethylamine) (Zaitsu et al. 2011).

Another group of synthetic cathinones is the pyrrolidinophenone-like family, which is characterized by a pyrrolidinyl substitution at the nitrogen atom (Westphal et al. 2007). These compounds are derivatives of α-PPP (Fig. 5). MPPP is the result of a ring methylation of α-PPP, and the alkylation at R_4 of MPPP produces MPBP, pyrovalerone, and MPHP. α-PVP results from the insertion of an ethyl group at R_4, while a 4-methoxy substitution in the ring of α-PPP leads to the formation of MOPPP.

A further α-PPP derivative was mentioned in the EMCDDA data base regarding drug profiling of synthetic cathinones. The substance is the 4-methyl-α-pyrrolidino-α-methylpropiophenone, resulting from a methylation of MPPP at R_5. It is the only cathinone derivative to have an alkyl substitution at this point, but no further information concerning this substance is available in the literature (http://www.emcdda.europa.eu/publications/drug-profiles/synthetic-cathinones).

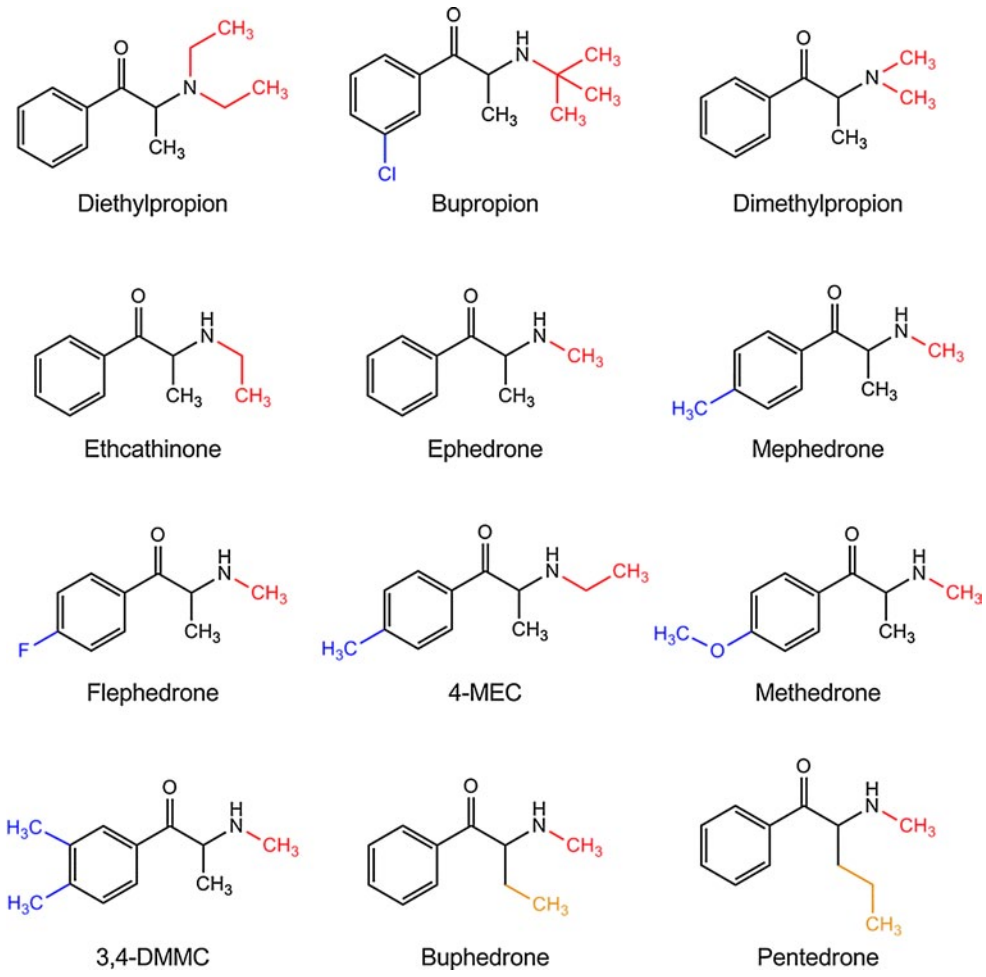

Fig. 3 Chemical structures of *N*-alkylated cathinone derivatives. *4-MEC* 4-methylethcathinone, *3,4-DMMC* 3,4-dimethylmethcathinone

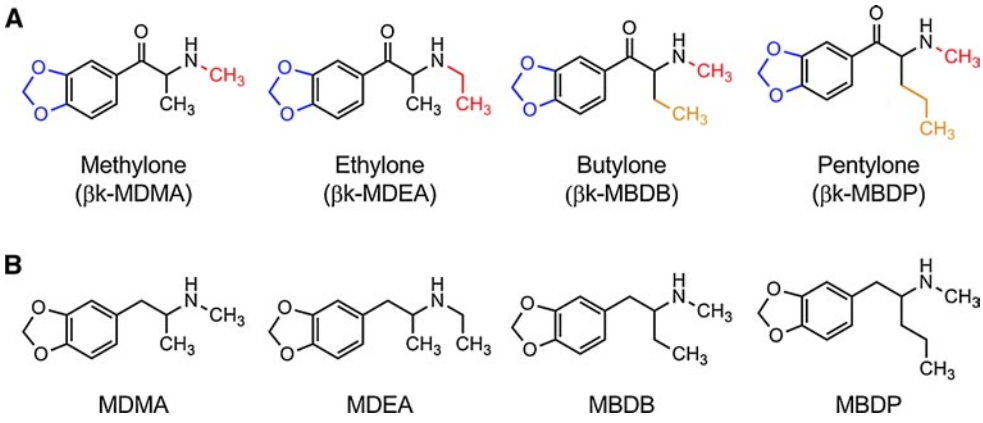

Fig. 4 Chemical structures of 3,4-methylenedioxy-*N*-alkylated cathinone derivatives (**a**) and related 3,4-methylenedioxyamphetamines (**b**). *MDMA* 3,4-methylenedioxymethamphetamine, *MDEA* 3,4-methylenedioxyethamphetamine, *MBDB* 3,4-methylenedioxy-α-ethyl-*N*-methylphenethylamine, *MBDP* 3,4-methylenedioxy-α-propyl-*N*-methylphenethylamine

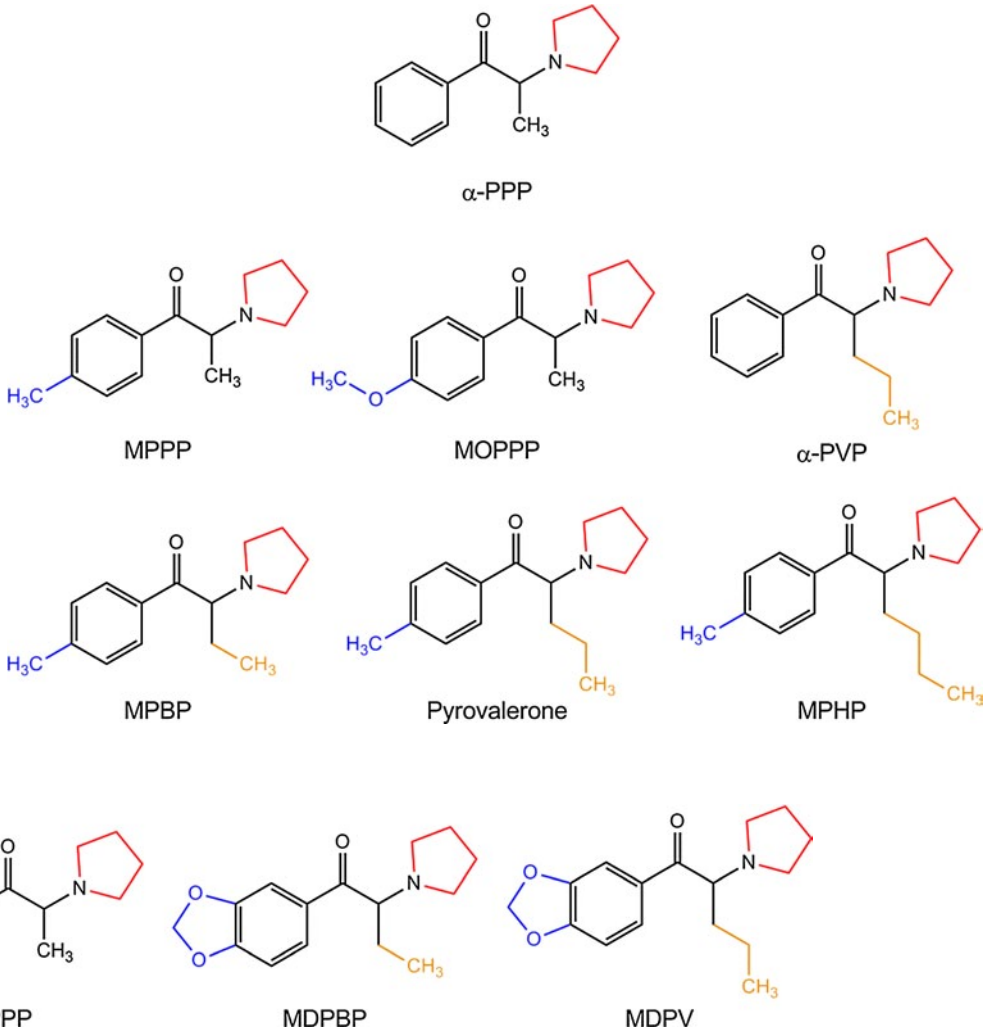

Fig. 5 Chemical structures of *N*-pyrrolidine cathinone derivatives. *α-PPP* α-pyrrolidinopropiophenone, *MPPP* 4-methyl-α-pyrrolidinopropiophenone, *MOPPP* 4-methoxy-α-pyrrolidinopropiophenone, *α-PVP* α-pyrrolidinovalerophenone, *MPBP* 4-methyl-α-pyrrolidinobutiophenone, *MPHP* 4-methyl-α-pyrrolidinohexiophenone

Fig. 6 Chemical structures of 3,4-methylenedioxy-*N*-pyrrolidine cathinone derivatives. *MDPPP* 3,4-methylenedioxy-α-pyrrolidinopropiophenone, *MDPBP* 3,4-methylenedioxy-α-pyrrolidinobutiophenone, *MDPV* 3,4-methylenedioxypyrovalerone

From the combination of these two last groups appears the synthetic cathinone family that has both the 3,4-methylenedioxy ring substitution and the *N*-pyrrolidinyl moiety (Kelly 2011), namely MDPPP, MDPBP, and MDPV (Fig. 6).

Naphyrone, a second-generation derivative presenting a naphthyl ring, displays a unique structural characteristic, not seen in any other reported synthetic cathinones so far. Brandt et al. (2010c) showed that NRG-1 products,

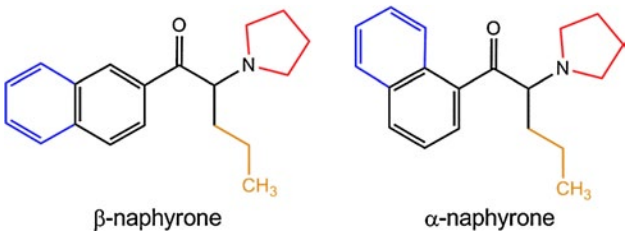

Fig. 7 Chemical structures of β- and α-naphyrone

generally advertised as containing naphyrone, may contain two isomers of this compound, namely β- and α-naphyrone (Fig. 7), and not only the β isomer, as it is regularly described in the literature.

Pharmacokinetics

In a single khat session, approximately 100–500 g of khat leaves is slowly chewed for several hours (Feyissa and Kelly 2008). Cathinone is the main active alkaloid present in the khat plant and was found to be present at around 78–343 mg per 100 g of fresh leaves (Arunotayanun and Gibbons 2012; Klein et al. 2012; Sakitama et al. 1995).

The psychostimulant effects induced by khat appear after approximately half an hour of chewing and last for about 3 h (Brenneisen et al. 1990; Kalix 1996). During this time, nearly 90 % of the alkaloids are efficiently released from the leaves. The absorption of these compounds occurs in two phases: the main route is through the oral mucosa, with 60 % of cathinone being efficiently absorbed, and the second route takes place in the stomach and small intestine after the juice has been swallowed (Arunotayanun and Gibbons 2012; Feyissa and Kelly 2008; Toennes et al. 2003; Toennes and Kauert 2002).

Wilder et al. (1994) determined a maximal plasma concentration of cathinone of 127 ± 53 ng/ml, attained 127 ± 30 min after ingestion of one dose of 0.8 mg/kg body weight. In another study, for one dose of 0.8–1 mg/kg, a mean peak of cathinone of 83 ng/ml is reached within 1.5–3.5 h (Halket et al. 1995). For a lower dose (0.6 mg/kg of body weight), Toennes et al. (2003) described a lower maximal plasma cathinone concentration (58.9 ± 18.8 ng/ml), attained at a comparable time after ingestion (2.31 ± 0.65 h). These results suggest that plasma concentration of cathinone, but not the time of peaking, is dependent on the dose ingested.

Less than 7 % of the ingested dose of cathinone appears unchanged in the urine, being mainly eliminated in the form of its metabolites, cathine, and norephedrine (Brenneisen et al. 1986; Toennes and Kauert 2002).

The doses of synthetic cathinones that are usually consumed may greatly vary from derivative to derivative, depending on the potency of their effects and the route of administration (Kelly 2011; Prosser and Nelson 2012). Furthermore, due to the variable nature of the contents of the purchased 'bath salts', regarding concentrations and purity of the products, the pharmacokinetics and pharmacodynamics inherent to actual exposure is rather unpredictable (Brandt et al. 2010a, b; Davies et al. 2010).

Cozzi et al. (1999) showed that methylone and EPH are less potent than their related non-keto phenylalkylamines, respectively, MDMA and methamphetamine (METH), regarding in vitro neurotransmitter uptake inhibition. Generally, the presence of the β-keto group increases the polarity of the synthetic cathinones, resulting in a decrease in their ability to cross the blood–brain barrier (BBB) (Hill and Thomas 2011; Lindsay and White 2012; Schifano et al. 2011). Consequently, to attain equipotent effects, the doses of synthetic cathinones reported by the users are usually higher than those reported for the related amphetamines, and for some derivatives, like MEPH, there is often the need to repeat the dose shortly after the first intake (Archer 2009; Deluca et al. 2009b; Kelly 2011; Prosser and Nelson 2012). This polarity issue occurs mainly with the *N*-alkylated derivatives, but not so much with the pyrrolidine family of cathinones, since the presence of the pyrrolidine ring greatly reduces the polarity of these compounds (Coppola and Mondola 2012). Nonetheless, methylone and MDPV, as well as EPH and MEPH, exhibited high permeability in human brain capillary endothelial cells expressing blood-to-brain and brain-to-blood transporters. Among the four derivatives, the BBB was most permeable to MDPV and MEPH in this order, and evidences suggest that the first one is actively transported into the brain via specific blood-to-brain influx carriers (Simmler et al. 2013).

Doses of MEPH between 20 and 50 mg are known to elicit some psychostimulant effects, but self-reported dosages for a typical session range from 500 mg to 1 g of powder (Deluca et al. 2009b; Prosser and Nelson 2012; Winstock et al. 2011). When insufflated, 20–75 mg of MEPH induces a rapid onset of effects, which last less than 2 h. Typical oral dosages range from 150 to 250 mg, with the onset of expected effects within 45 min after ingestion, and duration of action of almost 5 h. Intravenous administration of this derivative has a faster onset of stimulation, peaking 10–15 min after injection, and lasting for less than 30 min. Binging and mixing routes of administration in a single session is frequent and have the intent to achieve both rapid and long-lasting effects (Karila and Reynaud 2011; Mas-Morey et al. 2012; Prosser and Nelson 2012).

MDPV redosing is also common but, unlike MEPH, MDPV can induce stimulant effects at dosages as low as

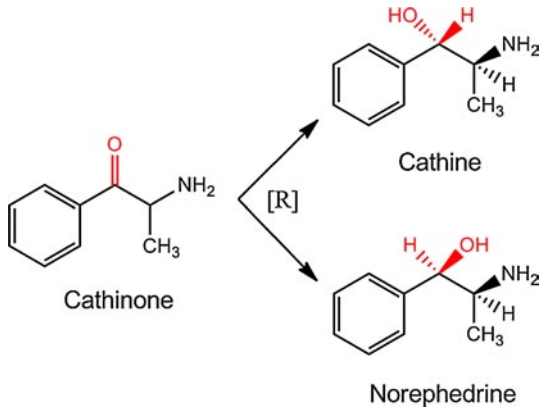

Fig. 8 Phase I metabolism of cathinone. [R], reduction [adapted from (Brenneisen et al. 1986)]

5 mg. Typical administered doses can go up to 20 mg or higher when redosing. Intakes of over 200 mg of MDPV at once have been reported; however, for doses higher than 15 mg, some users report extremely unpleasant 'come-down' effects (Deluca et al. 2009a). The onset of action and the potency of effects are similar to the two main routes of administration (oral and intranasal), and occur in less than 30 min after consumption. MDPV-induced stimulation lasts for 2–3.5 h after intranasal intake and up to 7/8 h when ingested (Deluca et al. 2009a; Karila and Reynaud 2011).

Methylone was primarily sold in 5-ml tubes, but is currently available in the form of powder or capsules. Common oral dosages range from 100 to 300 mg, with doses higher than 250 mg being considered a 'heavy' consumption. The effects are reported within 15–30 min, with the duration of over 3 h. However, some stimulating effects may be experienced during the 24 h following intake (Bossong et al. 2005; Karila and Reynaud 2011; Lopez-Arnau et al. 2013; Warrick et al. 2012).

Little information on butylone use is available in the literature. Online data of self-reported use suggest that usual doses of this derivative are similar to methylone, with stimulation lasting for 4–6 h (Karila and Reynaud 2011; Warrick et al. 2012).

The natural cathinone, like all synthetic cathinones, undergoes phase I metabolism after absorption, namely a reduction of the β-keto group to an alcohol catalyzed by liver microsomal enzymes (Brenneisen et al. 1986; Guantai and Maitai 1983), producing cathine and norephedrine (Fig. 8). In the specific case of cathinone, the metabolism was determined to be stereoselective, with the principal metabolite of the stereoisomer *S*-(−)-cathinone being norephedrine, whereas *R*-(+)-cathinone is metabolized into cathine (Mathys and Brenneisen 1992). The amount of norephedrine excreted in urine was found to be much higher than the amount ingested due to a combination of absorbed norephedrine (also present in khat leaves) and the product of cathinone metabolism (Toennes and Kauert 2002).

This stereoselective metabolism was also demonstrated for dimethylpropion and then proposed for EPH (Fig. 9) (Markantonis et al. 1986; Sparago et al. 1996). The reduction of EPH and dimethylpropion originates ephedrine and methylephedrine, respectively, which are further metabolized into norephedrine and ephedrine through a *N*-demethylation (Emerson and Cisek 1993; Paul and Cole 2001).

For MEPH, three phase I metabolic pathways were determined both in rat and human urine samples, with 7 metabolites being identified in the human samples. Besides the *N*-demethylation of the primary amine, MEPH can undergo oxidation in the ring methyl group, producing an alcohol that can be further oxidized to give a carboxylic acid and then reduced at the β-keto group (Fig. 10). The first metabolite resulting from MEPH *N*-demethylation may also be oxidized or reduced (Meyer et al. 2010b). Cytochrome P450 (CYP) 2D6 (CYP2D6) was found to be the main responsible enzyme for the phase I metabolism of MEPH in human liver microsomes (Pedersen et al. 2013).

Recently, Khreit et al. (2013) developed an in vitro method for the characterization of the phase I and II metabolic pathways of MEPH. Rat hepatocytes were incubated with MEPH for 2 h, after which the supernatant was analyzed by LC–MS. Seventeen metabolites were identified, from which 7 were phase II metabolites, resulting from the reactions of acetylation (Fig. 10a) and/or glucuronidation (Fig. 10b). Regarding the reduced metabolites, phase II metabolism at the hydroxyl groups may also be expected.

The metabolism of 3,4-methylenedioxy cathinones, including methylone, butylone, and ethylone, has been characterized (Kamata et al. 2006; Zaitsu et al. 2009): the three pathways being the *N*-dealkylation (minor pathway),

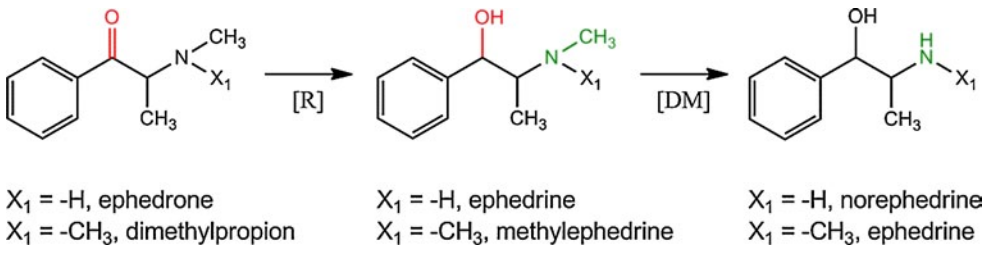

Fig. 9 Metabolism of ephedrone and dimethylpropion. [R], reduction; [DM], *N*-demethylation [adapted from (Markantonis et al. 1986)]

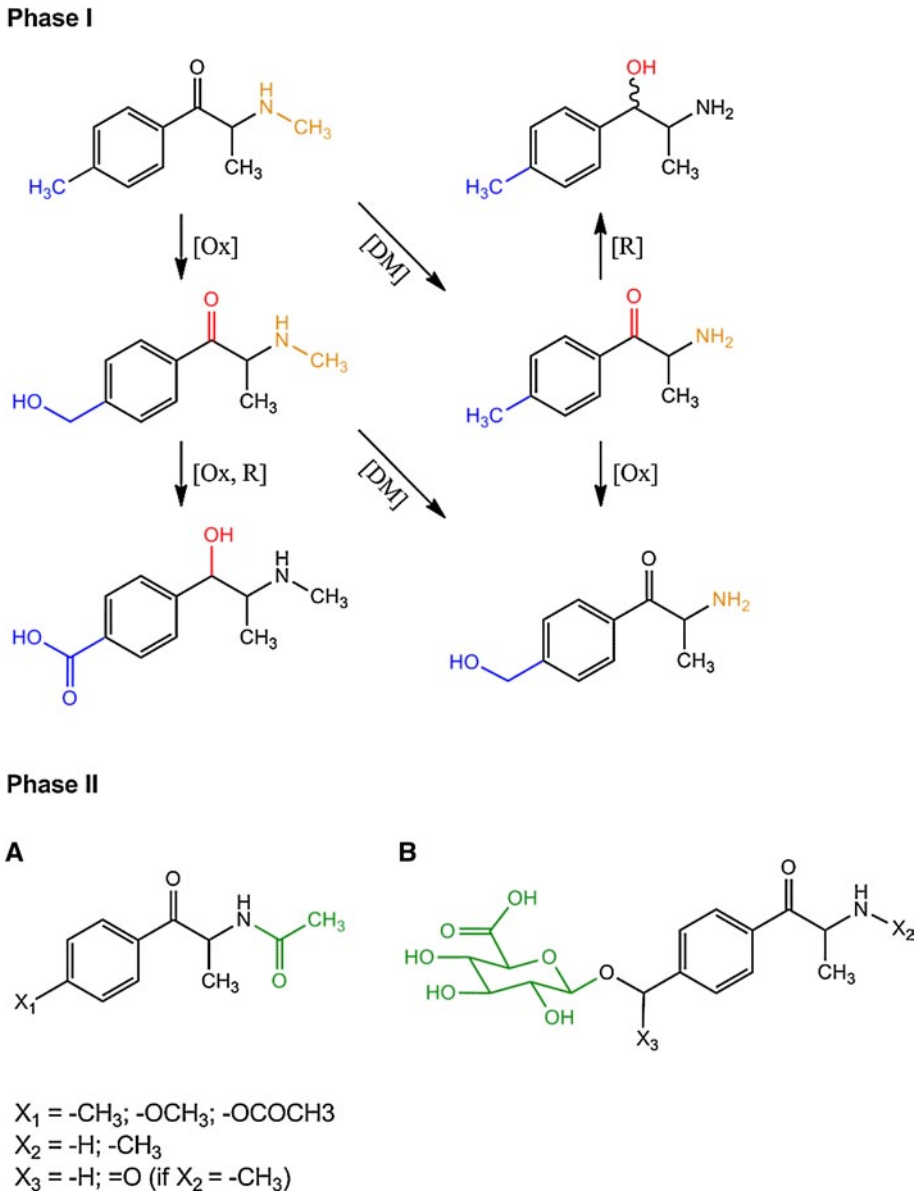

Fig. 10 Phase I metabolism of mephedrone and phase II metabolites. [R], reduction; [DM], *N*-demethylation; [Ox], oxidation [adapted from (Meyer et al. 2010b; Khreit et al. 2013)]

the reduction of the β-keto moiety, and finally, the demethylenation followed by a *O*-methylation mediated by the catechol *O*-methyltransferase (COMT) (Fig. 11). The three hydroxylated metabolites resulting from the two last pathways are more likely to undergo phase II metabolism, namely glucuronidation and sulfonation of the alcohol group, and the conjugates are excreted in the urine, along with the unmetabolized drugs (Coppola and Mondola 2012; Shima et al. 2009; Zaitsu et al. 2009).

Like in other synthetic cathinones, for pyrrolidine derivatives, such as MDPV and α-PVP, the ketone group in the side amine chain is converted into an alcohol (Fig. 12). Regarding MDPV, the 3,4-methylenedioxy ring is metabolized the same way as the βk-methylenedioxyamphetamines (see Fig. 11), producing the catechol and the methoxy catechol pyrovalerone. Strano-Rossi et al. (2010) determined these to be the main MDPV metabolites, which may be sulfonated or glucuronidated, and later excreted in the urine.

Demethylenation was also the main pathway established for the degradation of MDPPP in a metabolic in vitro study using human liver microsomes. Besides CYP2D6, the isoenzyme CYP2C19 was determined to be almost equally responsible for this reaction, from which results the metabolite di-OH-PPP (Springer et al. 2005).

Further biotransformation of the pyrrolidine group was proposed, specifically for MDPV and α-PVP (Meyer et al. 2010a; Sauer et al. 2009). For instance, the pyrrolidine ring can be degraded, producing primary amines. On the other hand, the side chain and the position 2′ of the pyrrolidine

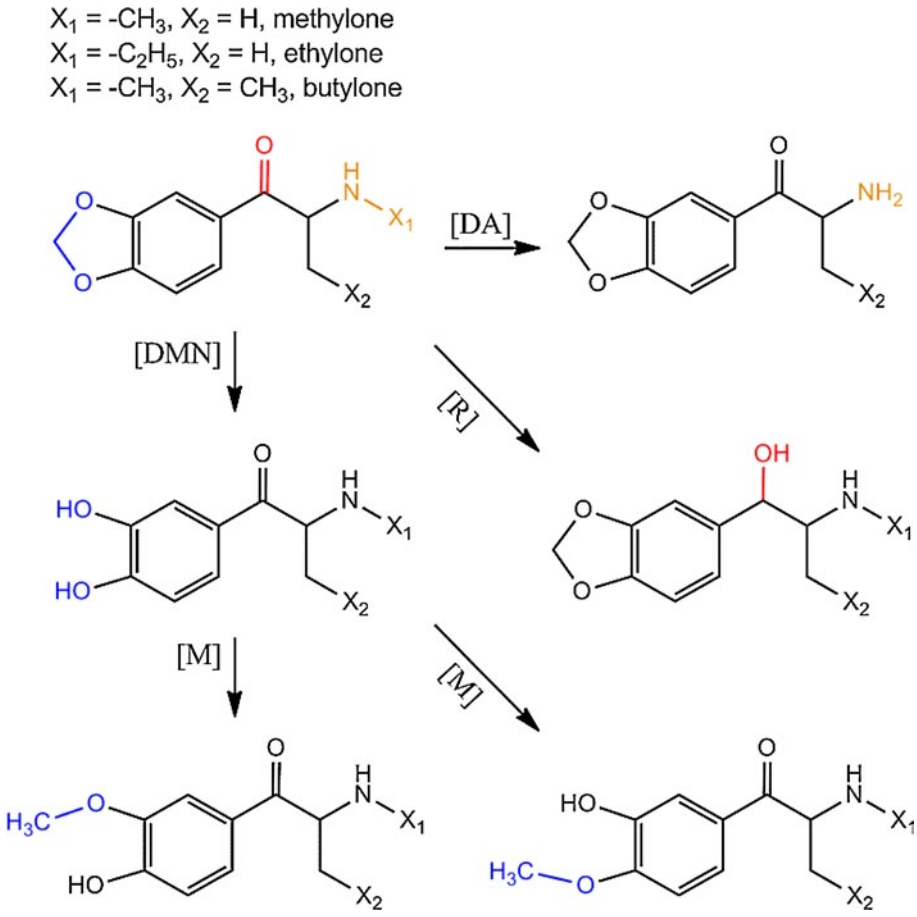

Fig. 11 Phase I metabolism of the 3,4-methylenedioxy-*N*-alkylated cathinone derivatives. [R], reduction; [DA], *N*-dealkylation; [DMN], demethylenation; [M], *O*-methylation [adapted from (Kamata et al. 2006; Zaitsu et al. 2009)]

ring may be hydroxylated, followed by dehydrogenation to a ketone and to a lactam, respectively. Finally, the ring can open to the corresponding aliphatic aldehyde and undergo further oxidation, producing a carboxylic acid (Fig. 12). In the particular case of α-PVP, the phenyl ring can be hydroxylated, most probably in the position 4′ (Sauer et al. 2009). The resulting metabolites, along with others detaining a hydroxyl group, can partially undergo phase II metabolism.

Similar metabolic pathways were recently proposed by Meyer et al. (2013) for β-naphyrone.

It is worth noticing that the metabolism of flephedrone is predictably slower than other synthetic cathinones, as fluorination often results in more stable compounds, and is consequently more resistant to enzymatic cleavage of the C–F bond (Westphal et al. 2010). Like α-PVP, phase I metabolism of this 'legal high' includes hydroxylation of the phenyl ring, besides the common β-keto reduction and *N*-demethylation to give a primary amine, as determined in rabbit liver slices and human liver microsomes (Meyer et al. 2012; Pawlik et al. 2012). *N*-demethylation of flephedrone is mainly mediated by the human CYP2B6 enzyme, but the isoenzymes 2D6, 2C19, 2E1, and 3A4 are able to catalyze this reaction too. Furthermore, the metabolite resulting from the β-keto reduction can be excreted as a glucuronic acid conjugate (Meyer et al. 2012).

Pharmacodynamics

As depicted in Fig. 13, cathinone is the β-keto analog of amphetamine, while its metabolites (cathine and norephedrine) are structurally closely related to noradrenaline. The chemical similarity between cathinone and amphetamine, and the amphetamine-like subjective effects of khat chewing, led to the designation of 'natural amphetamine' given to cathinone (Kalix 1992).

In fact, cathinone shares with amphetamine both CNS stimulant and sympathomimetic effects. Early studies on the pharmacological activity of the khat leaves showed that cathinone, cathine, and norephedrine are capable of inducing an amphetamine-like CNS dopamine release, with cathinone being the most potent of the three alkaloids (Kalix 1983; Kalix and Braenden 1985).

It is of note that, as for amphetamine, the presence of a methyl group at the α-position of the phenylethylamine side chain prevents the inactivation of cathinone, cathine,

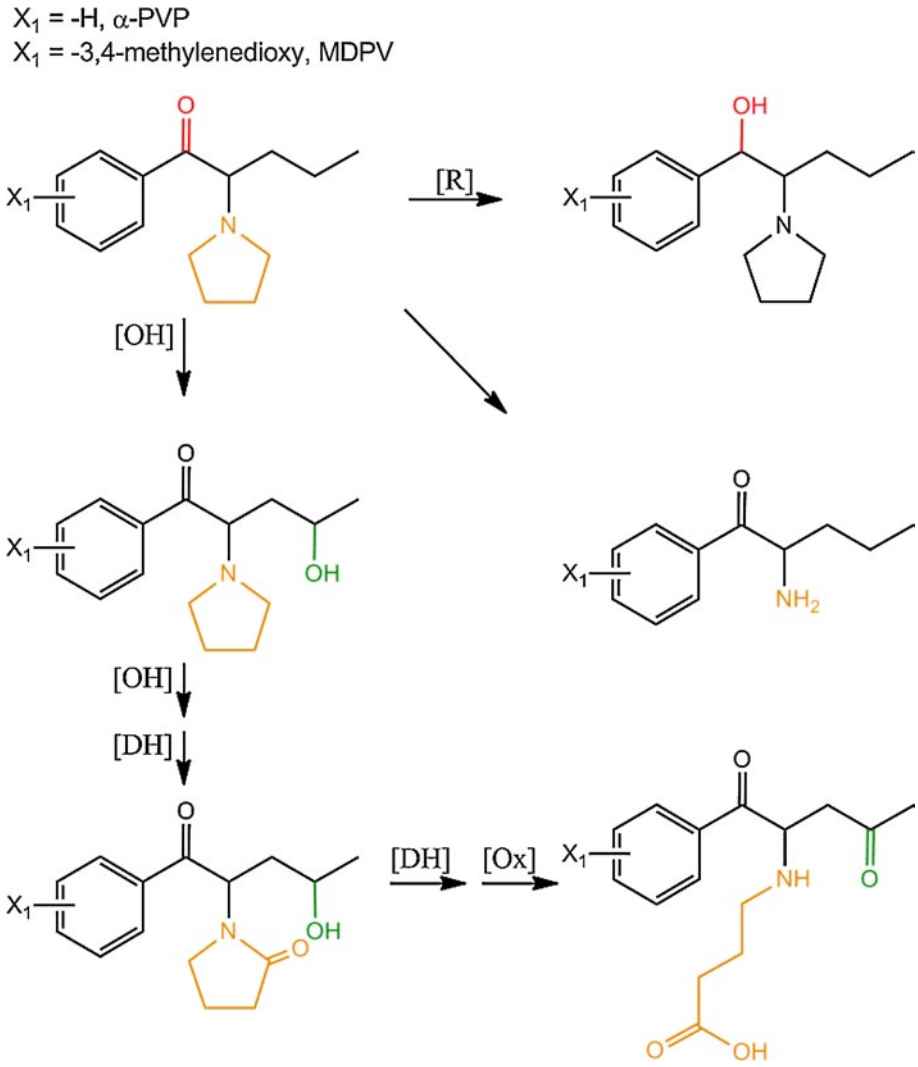

Fig. 12 Phase I metabolic pathways common to α-PVP and MDPV. [R], reduction; [OH], hydroxylation; [DH], dehydrogenation; [Ox], oxidation [adapted from (Meyer et al. 2010a; Sauer et al. 2009)]

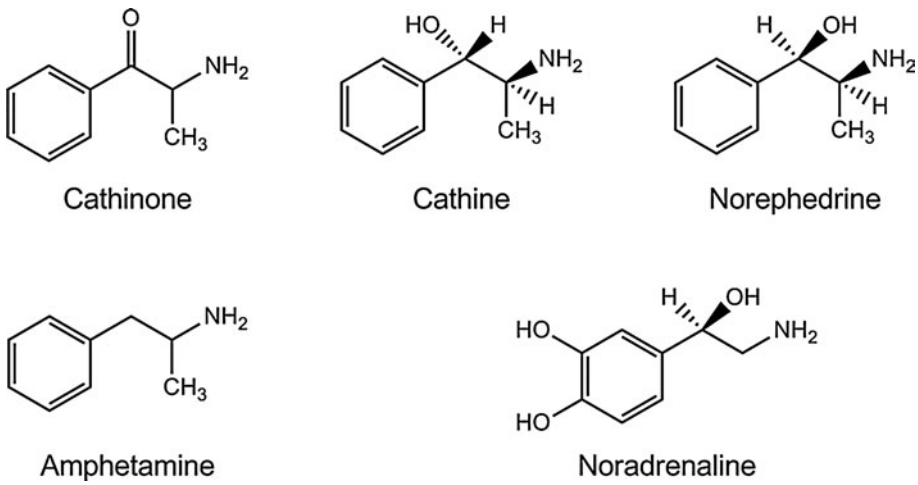

Fig. 13 Chemical structures of cathinone, cathine, norephedrine, amphetamine, and noradrenaline

and norephedrine through monoamine oxidase (MAO) (Siegel et al. 1999). Furthermore, it was shown that cathinone inhibits MAO more strongly than amphetamine (Nencini et al. 1984) and is more selective toward the isoenzyme MAO-B (Osorio-Olivares et al. 2004), whose inhibition leads to a decrease in dopamine degradation, and

consequently to the synaptic accumulation of this catecholamine. Additionally, cathinone was found to be more lipophilic than its metabolites, favouring its penetration into the CNS (Kalix 1991).

In view of the peripheral actions, khat chewing or cathinone administration is accompanied by a sympathomimetic syndrome, which is characterized by increased heart rate and blood pressure, mydriasis, and hyperthermia, among other effects. These effects suggest a mode of action similar to amphetamine, i.e., cathinone promotes release of catecholamines at nerve endings (Kalix 1992).

Since 'legal highs' are a rather recent matter of concern, and due to the shortage of reliable information in the literature on their molecular pharmacology, research regarding synthetic cathinones often resorts to comparisons with illicit drugs with similar subjective effects, namely amphetamine and cocaine. In fact, synthetic cathinones, likewise amphetamines [for review, see (Carvalho et al. 2012)], were shown to exert their effects by interacting with plasma membrane monoamine transporters, namely dopamine transporter (DAT), noradrenaline transporter (NET), and serotonin transporter (SERT), resulting in an increased concentration of these biogenic amines in the synaptic cleft (Baumann et al. 2012; Cameron et al. 2013; Cozzi et al. 1999; Lisek et al. 2012; Lopez-Arnau et al. 2012; Simmler et al. 2013; Sogawa et al. 2011). However, different affinities toward these transporters are observed between cathinone derivatives. When interacting with monoamine membrane transporters, drugs can be classified as either substrates (translocated into cells where they disrupt vesicular storage and stimulate non-exocytotic release of neurotransmitters by reversing the normal direction of transporter flux), like amphetamines, or blockers, like cocaine, which ultimately leads to different repercussions regarding long-term effects (Baumann et al. 2013a). Recent experiments with different study models (e.g., in vivo microdialysis in rats and in vitro rat brain synaptosomes and human embryonic kidney cells (HEK293 cell line) expressing DAT and SERT) showed that MEPH, methylone, ethylone, butylone, and naphyrone act as non-selective inhibitors for all catecholamine transporters and, with the exception of naphyrone, also as serotonin releasers, similar to MDMA (Baumann et al. 2012, 2013a; Simmler et al. 2013). Contrarily, MDPV was shown to induce powerful cocaine-like effects when tested in rat brain synaptosomes. MDPV acts as a pure monoamine-selective transporter blocker, with high potency for DAT and NET (50-fold and 10-fold more potent than cocaine, respectively), but weak for SERT (10-fold less potent than cocaine) (Baumann et al. 2013b; Cameron et al. 2013).

Similar mechanism of action was determined for pyrovalerone, whose structure is closely related to MDPV. Pyrovalerone and MDPV are very potent, and selective DAT and NET inhibitors, but unlike amphetamines, do not evoke monoamine release (Baumann et al. 2013a, b; Simmler et al. 2013).

EPH and flephedrone were shown to act as preferential dopamine and noradrenaline uptake inhibitors and dopamine releasers, similar to the mechanism of action of amphetamine and methamphetamine (Simmler et al. 2013).

In addition, all cathinones were shown to efficiently permeate an in vitro blood–brain barrier model, with MDPV exhibiting very high transmembrane permeability (Simmler et al. 2013).

Like other drugs of abuse, such as cocaine, MDMA, and METH, the affinity toward NET may be related to the sympathomimetic effects experienced with cathinone derivatives, while the potency to inhibit DAT may be associated with their psychostimulant effects and addictive potential. On the other hand, a greater affinity for SERT has been associated with the symptoms of paranoia and hallucinations, similar to those observed with classic hallucinogenic drugs, including psilocybin, mescaline, and LSD. Furthermore, symptoms of depression and anhedonia could be resultant from both serotonin and dopamine putative depletion induced by these compounds (Baumann et al. 2012; Cameron et al. 2013; Cozzi et al. 1999; Lisek et al. 2012; Lopez-Arnau et al. 2012; McGraw and McGraw 2012; Schifano et al. 2011; Simmler et al. 2013; Sogawa et al. 2011).

MEPH, one of the most abused synthetic cathinone worldwide, demonstrated to detain a unique psychostimulant profile, sharing pharmacological properties that appear to be similar to both METH and MDMA. On one hand, MEPH is capable of inducing MDMA- and cocaine-like subjective effects, which may contribute to its indiscriminate abuse (Carhart-Harris et al. 2011; Deluca et al. 2009b). On the other hand, it causes dopamine release at much greater levels than MDMA and comparable to those induced by METH. This may be problematic when regarding chronic and long-term consumption, as MEPH may present enhanced abuse liability, confirmed by its ability to elicit self-administration patterns in animal models (Hadlock et al. 2011), and the tendency for users to frequently binge on MEPH (Deluca et al. 2009b), just like METH.

Physiological and toxicological effects in animal studies

Khat and several synthetic cathinones have been shown to affect locomotor behavior in animals, but at variable potencies and different time course of actions (Marusich et al. 2012). Both acute and repeated oral administrations of *C. edulis* (200 mg/kg) or cathinone (15 mg/kg) significantly enhanced the locomotor activity and aggressive behaviors in male Sprague–Dawley rats (Banjaw et al. 2006).

Several studies have demonstrated MEPH's ability to elicit locomotor hyperactivity in rats and mice, with the vesicular serotonin content and DAT inhibition playing key roles in this effect (Lisek et al. 2012; Lopez-Arnau et al. 2012; Martinez-Clemente et al. 2013; Marusich et al. 2012; Miller et al. 2013; Motbey et al. 2012a; Shortall et al. 2013a; Wright et al. 2012a).

Similarly, methylone, butylone, flephedrone, 3-FMC, methedrone, and MDPV were shown to induce a dose-dependent hyperlocomotion on rats and mice too (Fantegrossi et al. 2013; Lopez-Arnau et al. 2012, 2013; Marusich et al. 2012), specially MDPV, which was found to elicit hyperactivity at intraperitoneal doses as low as 1 mg/kg, being 10-fold more potent than cocaine (Marusich et al. 2012).

Additionally, MEPH was also implicated in cognitive processes. In fact, MEPH administration improved visuospatial memory and learning processes in rhesus monkeys (Wright et al. 2012b), but significantly reduced the working memory performance in mice, and induced long-term memory impairment in rats (den Hollander et al. 2013; Motbey et al. 2012b).

Cardiovascular toxicity is a well-known feature of MEPH abuse. Meng et al. (2012) showed that this substance elicits significant increases in heart rate, blood pressure, and cardiac contractility in guinea pigs, but does not act directly as a pro-arrhythmic drug. Increases in heart rate and mean arterial pressure induced by MEPH were also determined in rats (Varner et al. 2013).

Some synthetic cathinones could be established as affecting thermoregulation in animal models. For instance, sustained and significant increases in rectal temperature were observed in male Lister hooded rats injected with cathinone or EPH (Shortall et al. 2013b), as well as in Wistar rats orally administered with cathinone (Tariq et al. 1989). Repeated administrations of methylone evoked significant hyperthermia in male Sprague–Dawley rats, with an increase of approximately 2 °C 5 h after intravenous injection, at a concentration as low as 3 mg/kg (Baumann et al. 2012). MDPV was also shown to induce a dose-dependent hyperthermia in mice, but only when administered in a warm ambient temperature of 28 °C (Fantegrossi et al. 2013).

Some inconsistency concerning the thermoregulatory properties of MEPH is found in the literature. On one hand, MEPH was found to induce a dose-dependent hyperthermia in Sprague–Dawley rats administered with four subsequent doses of 10 or 25 mg/kg (Hadlock et al. 2011) or three consecutive 3 or 10 mg/kg doses (Baumann et al. 2012), and in mice with a binge-like administration of 4 doses of 20 or 40 mg/kg. In this last study, when injecting the highest dose of MEPH, it was observed an immediate drop on the core body temperature, followed by a rapid return and exceeding the controls to a prolonged hyperthermic status (Angoa-Pérez et al. 2012). On the other hand, a single subcutaneous administration of MEPH (1–10 mg/kg) was shown to induce significant hypothermia in Wistar rats, especially at a low ambient temperature (Wright et al. 2012a). MEPH was also shown to induce a dose-dependent reduction in body temperature in male Sprague–Dawley rats at an ambient temperature of 20 °C, but not at 30 °C (Miller et al. 2013), as well as a transient decrease in rectal temperature in male Lister hooded rats at 19–22 °C (Shortall et al. 2013b). Taken together, these findings appear to be consistent with the conversion of MEPH-induced hypothermia at single doses to the hyperthermic effects after a binging session.

Similarly to MDMA and METH, MEPH causes a rapid decrease in striatal DAT and hippocampal SERT function. However, despite the serotonergic depletion, and unlike METH, a binge-like treatment in rats with this cathinone derivative does not elicit persistent dopaminergic deficits and thus does not cause toxicity at dopamine nerve terminals (Angoa-Pérez et al. 2012; Hadlock et al. 2011). Nonetheless, MEPH was shown to potentiate the neurotoxicity evoked by other illicit drugs, such as METH, amphetamine, and MDMA (Angoa-Pérez et al. 2013).

Few studies regarding potential neurotoxic effects of synthetic cathinones, other than MEPH, are available in the literature. One early study on EPH-induced effects in mice demonstrated that this derivative has no long-term effects on serotonin neurons, but evokes significant toxic effects on dopaminergic nerve endings (Sparago et al. 1996). On the other hand, methylone produces dose-related increases in extracellular dopamine and serotonin, but has no long-term effects in cortical or striatal amines (Baumann et al. 2012).

Finally, evidences from animal studies also support the reinforcing properties and abuse liability of synthetic cathinones, specifically with MEPH and MDPV, confirmed by their ability to elicit self-administration patterns in rats (Aarde et al. 2013a, b; Hadlock et al. 2011; Motbey et al. 2013; Watterson et al. 2012b). A dose-dependent reinforcer efficacy was also reported in rats administered with methylone (Watterson et al. 2012a).

Subjective effects and adverse toxic reactions in humans

Khat chewing is characterized by a rapid onset of psychostimulant effects. Users often describe increased energy and excitement, and euphoric sensations, which historically resulted in their use to treat the symptoms of melancholia and depression. Users also experience improved sense of alertness, enhanced self-esteem, and increased ability

to concentrate, associate ideas, and communicate, which greatly contributes to the social character of this tradition (Alem et al. 1999; Cox and Rampes 2003; Dhaifalah and Santavy 2004).

Unpleasant physical or psychological effects emerge right after users stop chewing the leaves, but symptoms like restlessness, anxiety, and hypnagogic hallucinations may be experienced also during the process of chewing (Balint et al. 2009; Cox and Rampes 2003; Granek et al. 1988).

Withdrawal symptoms are similar to those observed with amphetamine or cocaine, and include insomnia, depression, lack of concentration, aggressiveness, lethargy, and different types of psychosis (Al-Motarreb et al. 2002; Alem et al. 1999; Halbach 1972; Giannini and Castellani 1982; Kelly 2011).

Chronic use of khat is commonly followed by severe cardiac, neurological, psychological, and gastrointestinal complications, and several cases of khat-related deaths have been reported (Chapman et al. 2010; Corkery et al. 2011). Peripheral effects, such as increased blood pressure and heart rate, have been associated with enhanced risk of myocardial infarction and acute coronary vasospasm (Al-Motarreb et al. 2005; Ali et al. 2010; Alkadi et al. 2002). Esophagitis, gastritis, and oral mucosal keratosis are typical consequences of the astringency of khat leaves. It has been shown that khat chewing also delays gastric emptying and intestinal absorption, which may contribute to malnutrition (Al-Habori 2005; Al-Motarreb et al. 2002). Oral cancer, acute and chronic liver disease, and cirrhosis have also been associated with long-term consumption of *C. edulis* (Chapman et al. 2010; Fasanmade et al. 2007; Kassie et al. 2001; Peevers et al. 2010; Roelandt et al. 2011; Soufi et al. 1991).

Finally, the most common neurological features of chronic abuse include insomnia, depression, impaired working memory, and psychosis. Khat chewing may also precipitate psychosis in patients already predisposed or with preexisting psychiatric disorders (Al-Motarreb et al. 2010; Colzato et al. 2011; Coppola and Mondola 2012; Cox and Rampes 2003; Odenwald 2007).

Subjective effects may vary between synthetic cathinones, but are similar to those experienced with khat. General desired effects encompass mild euphoria, enhanced empathy, decreased sense of insecurity and hostility, and increased libido (Deluca et al. 2009b; Prosser and Nelson 2012; Yohannan and Bozenko Jr 2010). Users also report unwanted effects, such as sweating, nausea and vomiting, headaches and dizziness, vertigo, confusion and impaired short-term memory, muscle twitching, palpitations and tremor, tachycardia and hypertension, and ultimately anhedonia, depression with suicidal ideations, psychosis, tolerance, and dependence (Bentur et al. 2008; Coppola and Mondola 2012; Karila and Reynaud 2011; Prosser and Nelson 2012; Sammler et al. 2010).

Like khat, adverse clinical features associated with synthetic cathinones abuse commonly include psychiatric, neurological, cardiac, and gastrointestinal symptoms, and existent data are normally referent to MEPH abuse.

Hallucinations, paranoia, panic attacks, aggressiveness, chest pain, and seizures associated with 'bath salts'-induced intoxication are typical side effects reported to the AAPCC (2013). Similar effects were described to the UK National Poisons Information Services during the period of March 2009 and February 2010. In a total of 131 telephone inquiries regarding MEPH use, 24 % suffered from agitation or aggressiveness, and 22 and 13 % reported tachycardia and chest pain, respectively. Psychotic symptoms were also recurrent (14 %), among other usual signs of intoxication, such as headaches, nausea, palpitations, peripheral vasoconstriction, and convulsions (James et al. 2011).

Agitation was also the most commonly described side effect after MEPH use in a Scottish emergency department, followed by chest pain and paresthesia (Regan et al. 2011). Typical sympathomimetic features reported to the Swedish Poisons Centres include tachycardia, restlessness, mydriasis, hypertension, and anxiety (Europol–EMCDDA 2010).

Hyponatremia and hyperthermia are two well-known features among 'ecstasy' users. The first one is also occasionally associated with MEPH-induced intoxication, suggesting a mechanism of action similar to MDMA, i.e., increased serotonin-mediated antidiuretic hormone secretion, with consequent decrease of sodium concentration in the blood (Sammler et al. 2010; Wood et al. 2010a). A case of methylone-induced hyponatremia, following several episodes of seizures, was also reported (Boulanger-Gobeil et al. 2012).

Hyperthermia is a toxicological effect that has been associated with the consumption of different cathinone derivatives, including MEPH, methylone, butylone, methedrone, and particularly MDPV (Borek and Holstege 2012; Fröhlich et al. 2011; Garrett and Sweeney 2010; Levine et al. 2013; Lusthof et al. 2011; Penders et al. 2012; Regunath et al. 2012; Rojek et al. 2012; Warrick et al. 2012; Wikstrom et al. 2010).

Besides all the adverse reactions described so far, several other effects may be associated with intoxication by synthetic cathinones abuse, including acute liver failure, acute kidney injury and rhabdomyolysis, and also symptoms related to the serotonin syndrome, such as hypertension, hyperreflexia, and tremor (Borek and Holstege 2012; Carhart-Harris et al. 2011; Coppola and Mondola 2012; Dargan et al. 2011; EMCDDA 2011; Fröhlich et al. 2011; Mugele et al. 2012; Prosser and Nelson 2012; Vardakou et al. 2011; Warrick et al. 2012). Table 2 summarizes case

Table 2 Case reports of toxicity associated with khat and synthetic cathinones use

Year/local	Gender/age	Substances used	Substances detected	Clinical presentation	Progress and outcomes	Reference
1982/Ohio, USA	Male/23	Khat leaves	Urine: phenylpropanolamine	Manic-like psychosis, hyperactivity, hostility, pacing, rapid speech, shouting, increased libido, dizziness, thirst, mydriasis, hypertension	Symptoms reduced and ceased the following 5 h	Giannini and Castellani (1982)
1988/Petah Tikva, Israel	Male/45	Khat leaves	–	Severe depressive state with suicidal ideation, hallucinations	–	Granek et al. (1988)
	Male/46			Hallucinations, anxiety, mild situational reaction, personality disorder		
	Male/67			Paranoid schizophrenia, mild cognitive and affective deficit		
1996–2006/Bristol, United Kingdom	Male/28–41 (7 cases)	Khat leaves	–	Hepatocellular injury, canalicular cholestasis, cirrhosis, portal fibrosis	One death; one liver transplantation	Peevers et al. (2010)
2010/London, United Kingdom	Male and Female/28–40 (6 cases)	Khat leaves	–	Hepatitis with multilobular necrosis	Four orthotopic liver transplantations; two deaths	Chapman et al. (2010)
2010/Leuven, Belgium	Male/26	Khat leaves	–	Tachycardia, hypertension, aggressive behavior, impaired cognitive status, hallucinations, acute blindness, panic attacks, acute liver failure	Orthotopic liver transplantation	Roelandt et al. (2011)
2004–2005/Haifa, Israel	Male and Female/16–54 (34 cases)	Cathinone	–	Headache, vomiting, hypertension, tachycardia, dyspnea, chest pain, myalgia, nausea, pulmonary edema, myocardial ischemia, intracerebral hemorrhage	One patient required neurosurgical intervention for intracerebral hemorrhage	Bentur et al. (2008)
2005/Ontario, Canada	Male/36	Ephedrone	Urine: manganese (2804.3 nmol/24 h) Blood: manganese (897 nmom/l)	Decreased libido, excessive sleepiness, impaired fine motor skills, choking, manganese-induced Parkinsonism	Rehabilitation declined; pharmacotherapy with no effect	de Bie et al. (2007)

Table 2 continued

Year/local	Gender/age	Substances used	Substances detected	Clinical presentation	Progress and outcomes	Reference
2005/Paris, France	Female/29	Ephedrone, alcohol, bromazepam	Blood: alcohol (0.167 g/dl) Serum: bromazepam (8.89 mg/l), methcathinone (0.50 mg/l) and methylephedrine (0.19 mg/l) Urine: benzodiazepines, ephedrone (17.24 mg/l), ephedrine (11.60 mg/l), methylephedrine (11.10 mg/l)	Coma, mydriasis, hypotension	Favorable evolution over the following 24 h; patient sent to psychiatric evaluation	Belhadj-Tahar and Sadeg (2005)
2010/Dundee, United Kingdom	Female/15	Mephedrone, alcohol	Urine: mephedrone metabolites	Nausea, vomiting, altered mental status, hypotension, hyponatremia, encephalopathy	Electrolyte imbalance resolved over 24 h	Sammler et al. (2010)
2010/Edinburgh and Falkirk, United Kingdom	Male and Female/19–59 (20 cases)	Mephedrone, alcohol (60 % of the cases)	–	Agitation, hallucinations, paranoid delusions, aggressive or abnormal behavior	Symptoms responded in a predictable way to appropriate pharmacotherapy; one death	Mackay et al. (2011)
2010/London, United Kingdom	Male/22	Mephedrone	Urine: mephedrone Serum: mephedrone (0.15 mg/l)	Palpitations, anxiety, agitation, chest pain, 'blurred tunnel vision', sweating, mydriasis, hypertension, tachycardia	Favorable evolution over the following 4 h	Wood et al. (2010b)
2010/London, United Kingdom	Male/16–36 (7 cases)	Mephedrone	Serum: mephedrone	Agitation, palpitations, chest pain, seizure, headaches, tachycardia, hypertension, hyponatremia	One death	Wood et al. (2010a)
2010/Dublin, Ireland	Male/19	Mephedrone	–	Chest pain, acute myocardial edema, acute myocardial inflammation	Discharged 5 days after admission	Nicholson et al. (2010)
2010/The Hague, the Netherlands	Male/36	Mephedrone	Femoral blood: mephedrone (5.1 mg/l), cocaine (0.0071 mg/l), MDMA (0.011 mg/l) Urine: mephedrone (186 mg/l) Stomach contents: mephedrone (1.04 g/l)	Severe agitation, aggression, hallucination, psychosis, hyperthermia	Fatal excited delirium	Lusthof et al. (2011)

Table 2 continued

Year/local	Gender/age	Substances used	Substances detected	Clinical presentation	Progress and outcomes	Reference
2012/Colchester, United Kingdom	Male/30	Mephedrone	–	Painful neck, upper chest swelling and mild trismus, odynophagia, subcutaneous emphysema in the head and neck	Discharge 48 h after admission	Maan and D'Souza (2012)
2010–2011/North Carolina, USA	Male/31	'Bath salts'	–	Confusion, aggressive behavior, paranoid delirium, dehydration, acute renal failure, hyperkalemia, rhabdomyolysis	Renal failure and rhabdomyolysis resolved over 3 days with supportive treatment	Penders et al. (2012)
	Male/30			Paranoia, agitation, violent behavior, abrupt change in mental status, acute renal failure, rhabdomyolysis, ARDS	Neurological status markedly improved within hours	
	Male/26			Fear, confusion, aggressive behavior, diaphoresis, hyperthermia	–	
2011/Mississippi, USA	Male/19	'Bath salts'	Urine: MDPV	Hallucinations, anxiety, paranoia, mild hypertension	–	Kyle et al. (2011)
2011/Louisiana, USA	Female/34	'Bath salts', cocaine, opiates	–	Necrotizing fasciitis at the site of injection, local infection	Surgical debridement and exploration	Russo et al. (2012)
2011/Virginia, USA	Male/late-20′s	'Bath salts'	–	Agitation, anxiety, paranoia, fear, suicidal and homicidal ideation, tachycardia, hypertension	Mental status normalized 30 h after 'bath salts' intake	Gunderson et al. (2013)
2011/Florida, USA	Female/29	'Bath salts'	Urine: benzodiazepines, cocaine	Altered mental status	Discharged with close psychiatry follow-up	Falgiani et al. (2012)
2011/California, USA	Male/22	'Bath salts', cannabis	Urine: cannabis	Severe chest pain, anxiety, nausea, hallucinations, diaphoresis, tachycardia	Symptoms resolved within 48 h after discharge	Striebel and Pierre (2011)
2011/Arizona, USA	Male/32	'Bath salts'	–	Insomnia, auditory hallucinations, paranoia, restlessness	–	Goshgarian et al. (2011)

Table 2 continued

Year/local	Gender/age	Substances used	Substances detected	Clinical presentation	Progress and outcomes	Reference
2012/Connecticut, USA	Male/26	'Bath salts'	–	Strange behavior, agitation, paranoia, diaphoresis, hallucinations, confusion, hypertension, tachycardia, acute kidney injury	Discharged to a psychiatric unit	Adebamiro and Perazella (2012)
2012/Missouri, USA	Male/39	'Bath salts'	–	Aggressive behavior, vomiting, hyperthermia, hypertension, tachycardia, renal failure with acute tubular necrosis	Patient discharged after continuous renal replacement therapy for 48 h	Regunath et al. (2012)
2012/Virginia, USA	Male/28	'Bath salts'	–	Confusion, altered level of consciousness, agitation, disorganized behavior, hallucinogenic delirium, psychosis	Discharged 3 days after admission	Sharma et al. (2012)
2012/California, USA	Male/29	'Bath salts'	Urine: cannabis	Psychosis, tangential thought process, erratic behavior, paranoid delusion, hallucinations	Psychotic symptoms resolved slowly over the following month	McClean et al. (2012)
2012/Ohio, USA	Male/38	'Bath salts'	Urine: benzodiazepines	Hallucinations, tachycardia, agitation, anxiety, paranoia, hostility	Improvements over the following day	Kasick et al. (2012)
	Male/26		–	Auditory hallucinations, paranoid delusions, feelings of detachment and derealization, suicidal ideation, tremors, delirium, confusion, hyperreflexia, myoclonus, amnesia, mild hypertension, tachycardia	Improvements 96 h after ingestion	
2013/California, USA	Male/18	'Bath salts'	Urine: α-PVP	Agitation, tachycardia, hyperthermia, apnea	Complete recovery	Levine et al. (2013)
	Male/37		Urine: MDPV Serum: MDPV (120 ng/l and 89 ng/l, 7 and 10 h after seeking medical care)	Agitation, tachypnea, hyperthermia, oliguric renal failure	Recovery 5 months later, remaining on hemodialysis	

Table 2 continued

Year/local	Gender/age	Substances used	Substances detected	Clinical presentation	Progress and outcomes	Reference
	Male/43		Urine: α-PVP	Altered mental status, agitation, tachypnea	Discharged, neurologically intact; decreased sensation to light touch involving the bilateral paraspinal muscles	
2013/Michigan, USA	Male/33	'Bath salts'	–	Tremor, anxiety, paranoia, mood instability, hypertension, agitation	Intermittent mild paranoia remained after discharge	Winder et al. (2013)
2013/Texas, USA	Female/19	'Bath salts'	–	Severe agitation, aggressive behavior, and psychosis, auditory and visual hallucination, tachycardia, hypertension, diaphoresis, mild mydriasis	Symptoms resolved after 48 h	Khan et al. (2013)
2012/Quebec, Canada	Female/22	Methylone, ethcathinone	–	Vomiting, tonicoclonic seizures, euphoria, agitation, sweating, thirst, disorientation, hyperreflexia, bruxism, hyponatremia	Discharged asymptomatic 6 days after arriving	Boulanger-Gobeil et al. (2012)
2012/Michigan, USA	Female/24	Methylone, butylone	Urine: methylone, butylone	Coma, hyperthermia, hyperreflexia, tachycardia, tachypnea	MOF, ARDS, renal failure, death	Warrick et al. (2012)
2013/California, USA	Male/19	Methylone	Blood: methylone (0.07 mg/dl)	Collapse	Death	Carbone et al. (2013)
2011/Pennsylvania, USA	Female/27	MDPV	–	Delusion, hallucinations, tachycardia, diaphoresis	Stabilization by the fifth day	Antonowicz et al. (2011)
	Male/32			Hypertension, tachycardia, insomnia	Discharged with no medications	
2012/Virginia, USA	Male/25	MDPV	Urine: MDPV (140 ng/ml)	Severe agitation, increased heart rate, hyperthermia, renal failure, fulminant hepatic failure, tachycardia, mydriasis, DIC, rhabdomyolysis	Discharged on day 18, requiring hemodialysis for the following month	Borek and Holstege (2012)

Table 2 continued

Year/local	Gender/age	Substances used	Substances detected	Clinical presentation	Progress and outcomes	Reference
2012/California, USA	Male/22	Flephedrone, MDPV	Urine: flephedrone (257 ng/ml), MDPV (136 ng/ml) Serum: flephedrone (346 ng/ml), MDPV (186 ng/ml)	Hallucinations, bizarre behavior, suicidality, tachycardia, mydriasis	Discharged asymptomatic 8 h after arriving	Thornton et al. (2012)
2010/London, UK	Male/31	Butylone and MDPV	Serum: butylone and MDPV	Tachycardia, insomnia, sweating, abdominal discomfort, anxiety, agitation, hypertension, mydriasis	Asymptomatic 4 h after admission	Wood et al. (2011)
2010/Ireland, UK	Male/28	Butylone and MDPV	–	Tonicoclonic seizure, hypertension, hyperthermia, tachycardia, sweating, rhabdomyolysis, acute renal and liver failure	Discharged 4 days post-admission to a psychiatric unit	Fröhlich et al. (2011)
2012/Krakow, Poland	Male/21	Butylone	Blood: butylone (20 mg/l)	Disorientation, hyperthermia, tachycardia, hypertension, increased muscle tonus, mydriasis, sialorrhea, hemorrhagic diathesis	Cardiac arrest with asystole and death	Rojek et al. (2012)
2011/Basel, Switzerland	Male/31	Naphyrone	Plasma: naphyrone (0.03 and 0.02 mg/l 40 and 60 h after intake, respectively)	Restlessness, blurred vision, agitation, insomnia, hallucinations, mortal fear, delusional psychosis, mydriasis	Reduced symptoms over the following day; accelerated thoughts and subtle delusional thought contents when self-discharged	Derungs et al. (2011)
2013/Texas, USA	Male/34	Naphyrone	–	Restlessness, agitation, hallucinations, mydriasis, bruxism, hypertension, tachycardia	–	Fay and Eitel (2013)

reports concerning khat and synthetic cathinones-induced intoxication.

Concluding remarks

Chewing khat leaves for their psychostimulant effects has been a social and cultural habit among Saudi Arabian and East African communities, for several centuries. Cathinone is the main psychoactive substance found in the leaves of the *C. edulis*. Chemically, cathinone is the β-keto analog of amphetamine, thus detaining similar main subjective effects, namely euphoria and increased energy. Unwanted clinical effects following khat abuse often include hallucinations, agitation, and anxiety. Chronic abuse may lead to severe neurological, cardiovascular, and gastrointestinal complications, including depression, psychosis, myocardial infarction, and acute liver failure.

Structurally modified cathinone derivatives were primarily synthesized for clinical use, mainly as antidepressants and anorectic agents, but promptly started being misused. Synthetic cathinones reached the recreational drug markets and have been sold as 'bath salts', as legal alternatives to illicit drugs like 'ecstasy' and cocaine, being conveniently available online and locally, at the so-called smartshops. So as to avoid legal control measures, these substances are labeled 'not for human consumption' and sold under deceptive labels lacking information on the product contents or on their potential harm for human health.

Over the last decade, it has been observed a great increase in the popularity of these 'legal highs', with over 30 synthetic derivatives having been identified so far. Information on their pharmacological and toxicological properties is scarce, but several 'bath salts'-induced intoxication cases and deaths have been reported worldwide.

MEPH, methylone, and MDPV are currently the main constituents of 'bath salts', but since their scheduling as controlled substances and criminal penalization, they have been gradually replaced by other derivatives with minor structural modifications to the natural cathinone.

In vivo data using animal models and in vitro methods have been crucial for the determination of the pharmacokinetics and pharmacodynamics of these compounds, as well as to elucidate the pharmacological and toxicological effects of the synthetic cathinones.

A rapid onset of effects is observed for the majority of the synthetic cathinones, with duration and potency greatly varying between derivatives. Following absorption, synthetic cathinones undergo phase I metabolism, with CYP isoenzymes mediating the main pathways. Phase II metabolic pathways may include reactions of acetylation, glucuronidation or sulfonation of the hydroxyl groups, with the resultant metabolites being excreted in the urine.

Like amphetamine, METH, and MDMA, synthetic cathinones exert their effects by interacting with catecholamine transporters (DAT, NET, and SERT), which results in increased synaptic concentrations of these monoamines. However, the selectivity of synthetic cathinones for these transporters varies considerably.

In vivo studies have also shown that synthetic cathinones are able to elicit locomotor hyperactivity, increased heart rate and hypertension. Some of them detain hyperthermic properties, as well. Evidences also support the reinforcing properties and abuse liability of MEPH, methylone, and MDPV.

Like khat, synthetic cathinones induce euphoric and empathogenic effects. Likewise, chronic abuse may result in adverse effects such as anxiety, agitation, hallucinations, paranoid delusions, tachycardia, hypertension, hyperreflexia, and eventually lead to acute liver and kidney failure and rhabdomyolysis.

Although many synthetic cathinones are currently under legal control in the EU state members and in most US states, criminalization of all cathinone derivatives is hard to attain. Some of them, like MEPH, have acquired a significant place in the illicit drug markets and still remain a matter of public concern. Additionally, several new derivatives emerge in the recreational legal markets every year.

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Hepatotoxicity and neurotoxicity of β-keto amphetamines: possible underlying cellular mechanisms

Clinical evidence has established liver damage and CNS injury as common complication following cathinones abuse (Borek and Holstege 2012; Carbone et al. 2013; Gavriilidis et al. 2015; Hohmann et al. 2014; Kramer et al. 2016; Murray et al. 2012; Omer and Doherty 2011; Ross et al. 2012; Valsalan et al. 2017), and *postmortem* studies further confirmed bioaccumulation in these target organs (Cawrse et al. 2012; Marinetti and Antonides 2013; McIntyre et al. 2013; Sykutera et al. 2015).

A limited number of *in vitro* hepato- and neurotoxicity studies on β-keto amphetamines have been reported in recent past years, showing a general increase in cell death in a concentration- and time-dependent manner (Araujo et al. 2015; den Hollander et al. 2014; den Hollander et al. 2015; Matsunaga et al. 2017; Nakagawa et al. 2009; Rosas-Hernandez et al. 2016a; Wojcieszak et al. 2016). However, the cellular mechanisms underlying the toxic potential of cathinones towards the brain and the liver are still not completely understood.

The putative mechanisms of cytotoxicity elicited by synthetic cathinones in these target organs will be presented and discussed in the following sections. In the lack of experimental evidence, we will conduct a parallel comparison of what may be expected from these new substances to what is widely known for amphetamine derivatives.

Long-term neurotransmitter depletion and oxidation of biogenic amines

β-Keto amphetamines exert their psychostimulant effects by interacting with membrane neurotransmitter transporters at the CNS, including noradrenaline (NA), serotonin (5-HT or 5-hydroxytryptamine) and dopamine (DA) transporters (NAT, SERT and DAT, respectively). Some cathinone derivatives act as relatively non-selective substrates for monoamine transporters, evoking transporter-mediated release of catecholamines into the synaptic cleft by reversing the normal transporter flux (Fleckenstein et al. 2007). This is the case of 4-MMC and methylone, which produce dose-related increases in extracellular DA and 5-HT *in vivo*, and an *in vitro* DA and NA releasing ability comparable to MDMA but inferior to methamphetamine (Baumann et al. 2012). Some cathinones act as pure transporter blockers like cocaine, with low to no monoamine release potential. This includes the pyrrolidine derivatives MDPV, pyrovalerone and α-pyrrolidinovalerophenone (α-PVP) as well as non-pyrrolidine derivatives such as pentedrone, which present strong NAT and DAT inhibitory activity, and minimal effects on SERT (Baumann et al. 2013b; Marusich et al.

2014; Simmler et al. 2013a; Simmler et al. 2014). Interestingly, MDPV was shown to be much more potent than cocaine with regards to the inhibition of transporter-mediated uptake *in vitro* (11- and 51-fold more effective on NAT and DAT inhibition in rat brain synaptosomes, respectively) and the ability to increase the extracellular levels of dopamine *in vivo* (10-fold more effective in the nucleus accumbens of conscious rats) (Baumann et al. 2013b). There are also cathinone derivatives that present a mixed mode of action, such as 4-methylethcathinone (4-MEC), which functions as SERT substrate/5-HT releaser and NAT and DAT blocker (Saha et al. 2015; Simmler et al. 2014). Either by reversing the flux of catecholamines, or by blocking their reuptake, synthetic cathinones promote the accumulation of DA, 5-HT and/or NE in the synaptic cleft, which results in sympathetic overstimulation due to increased binding of these neurotransmitters to postsynaptic receptors. The greater affinity towards DAT, comparatively to SERT, renders MDPV and related compounds a more stimulant profile and appears to contribute to higher reinforcing properties and greater propensity for abuse and addiction (Aarde et al. 2015; Baumann et al. 2016; Bonano et al. 2014; Eshleman et al. 2017; King et al. 2015; Simmler et al. 2013b; Watterson et al. 2014). In contrast, cathinone derivatives that present a lower DAT/SERT ratio on transporter inhibition, such as methylone and 4-MEC, are more empathogenic substances and appear to have lower abuse potential (Baumann et al. 2016; Creehan et al. 2015; Eshleman et al. 2017; Simmler et al. 2014; Watterson et al. 2012). Notwithstanding, other factors may contribute for the overall abuse potential of β-keto amphetamines, including their lipophilicity and pharmacokinetics. In fact, despite its low DAT/SERT ratio in comparison to METH, MDPV or even methylone (Baumann et al. 2012; Simmler et al. 2013a), the ability of 4-MMC to substitute for METH or cocaine in discriminative stimulus effects, to induce conditioned place preference and to sustain a behavior of self-administration, support rewarding and reinforcing properties, as well as a great abuse potential for this derivative (Bonano et al. 2014; Creehan et al. 2015; Gatch et al. 2013; Hadlock et al. 2011; Lisek et al. 2012; Motbey et al. 2013; Vandewater et al. 2015). This reflects in the highly addictive properties and uncontrolled bingeing behavior reported by 4-MMC users (Deluca et al. 2009b; Freeman et al. 2012; Schifano et al. 2011).

The neurotoxicity of amphetamines has been associated to the loss of DAT and SERT activity and the long-term depletion of DA and 5-HT (Cadet et al. 2007; Fleckenstein et al. 2007). There are a few studies regarding the neurotoxic effects of synthetic cathinones, which collectively present mixed results. For instance, persistent reductions of striatal DAT density were observed in abstinent methcathinone users, with levels comparable to those of users of its non-keto analogue, METH (McCann et al. 1998). Methcathinone was also shown to have the potential to damage both DA and 5-HT neurons, with evident differences between species and enantiomers (Sparago et al. 1996), and to

decrease the activities of tyrosine hydroxylase (TH) and tryptophan hydroxylase, the rate-limiting enzymes for the synthesis of DA and 5-HT, respectively, with consequent decline in the levels of these two neurotransmitters and their metabolites in rat striatum (Gygi et al. 1997). The work of Hadlock et al. (2011) revealed that repeated administration of 4-MMC in rats causes a significant decrease in the striatal DAT and hippocampal SERT functions, and a persistent serotonergic, but not dopaminergic, deficit. Accordingly, methylone administration also lead to a persistent decline of 5-HT in the frontal cortex, striatum and hippocampus of binge-treated rats, to levels comparable to those of amphetamine, but no significant effects were found in DA levels, neither for any monoamine in mice (den Hollander et al. 2013). In contrast, Baumann et al. (2012) showed that, contrarily to MDMA, neither 4-MMC nor methylone exhibit long-term changes in cortical or striatal amines after repeated administrations in rats. 4-MMC was also shown to lack neurotoxic effects towards dopaminergic nerve endings of the striatum of binge-treated mice (Angoa-Perez et al. 2012). However, 4-MMC enhanced the neurotoxic effects of amphetamine, METH and MDMA in the same model, with a significant decrease in striatal levels of DA, DAT and TH (Angoa-Perez et al. 2013), which raises concerns on the potentially dangerous interaction of synthetic cathinones with drugs that are commonly co-abused. Interestingly, despite the absence of prolonged neurotransmitter deficit in some of these studies, significant long-term cognitive and neurochemical effects were determined after 4-MMC administration in rats and mice (den Hollander et al. 2013). The underlying mechanisms for an apparent persistent effect of β-keto amphetamines in SERT and lack of long-term toxicity on the DA system remain largely unknown.

Long-term dopaminergic and serotonergic toxicity induced by amphetamines has been associated with their ability to interfere with the vesicular uptake of monoamines, by acting as a substrate of the vesicular monoamine transporter (VMAT), in particular VMAT2, which is the isoform expressed in CNS neurons (Erickson et al. 1996). Amphetamines act by competing with the neurotransmitters for VMAT2-mediated uptake or reversing the normal transporter flux, or through dissipation of the vesicular transmembrane pH difference that drives the uptake, as a consequence of the nonionic diffusion of amphetamine derivatives into the vesicles (Fleckenstein et al. 2007; Rudnick and Wall 1992; Schuldiner et al. 1993; Sulzer et al. 2005). Evidence suggest that β-keto amphetamines also detain the ability to inhibit VMAT2-mediated transport of 5-HT, NAT and DAT, though with a general lower affinity and potency than those of classical amphetamines (Asser et al. 2016; Cozzi et al. 1999; Eshleman et al. 2013; Lopez-Arnau et al. 2012; Pifl et al. 2015). For instance, Cozzi et al. (1999) showed that methcathinone and methylone were over 10-fold less potent than their non-keto analogues METH and MDMA, respectively, regarding the inhibition of 5-HT uptake via VMAT2 in bovine chromaffin granules. Eshleman et al. (2013) assessed

the ability of different cathinone derivatives to inhibit the vesicular uptake of 5-HT and to stimulate the release of NA at the human VMAT2 (hVMAT2) in transfected HEK cells, in a comparison study with METH and MDMA. Results showed that all cathinones were substantially weaker than the amphetamine analogues, with effects occurring at high micromolar concentrations. Butylone, 4-MMC and methylone were also shown to significantly inhibit VMAT2-mediated uptake of DA in an *in vitro* model of rat striatal synaptosomes, with 4-MMC being 6- and 24-fold stronger than methylone and butylone, respectively, with a half maximal inhibitory concentration (IC_{50}) of 3.40 µM (Lopez-Arnau et al. 2012) . However, when assessing the uptake of DA by hVMAT2 in a human striatum model, the IC_{50} of 4-MMC rises to 223 µM, 11-fold higher than MDMA (Pifl et al. 2015). Taken together, these data suggest that the inhibition of vesicular uptake of neurotransmitters by synthetic cathinones, though possible, may not be significant at physiological concentrations. Nevertheless, the contribution of this mechanism of action to the overall toxicity of β-keto amphetamines should not be disregarded.

Reduced VMAT2 uptake leads to a rise of unsequestered catecholamines within the presynaptic cytoplasm. Neurotransmitter turnover within the presynaptic terminal is balanced by its synthesis, release and metabolic inactivation. Monoamine oxidase (MAO) is a mitochondrial enzyme responsible for the first step of catecholamines deamination and consequent inactivation, with formation of aldehydes (Eisenhofer et al. 2004). Like amphetamines, the methyl substitution at the α-carbon present in several cathinone derivatives may hamper their deamination through this enzyme (Nencini et al. 1984), a mechanism partially accountable for amphetamine's prolonged action in the CNS (Liechti 2015). Furthermore, amphetamines also function as MAO inhibitors, hindering catecholamines inactivation and consequently increasing their availability for release (Steinkellner et al. 2011). Cathinone was determined to inhibit MAO with higher potency than amphetamine (Nencini et al. 1984), and Osorio-Olivares et al. (2004) showed that a series of aryl substituted cathinones, including 4-MMC, also exhibit MAO inhibitory activity. Nevertheless, the role of MAO inhibition by synthetic cathinones on drug-induced toxicity is yet to be fully elucidated. A general representation of the putative pharmacological mechanisms of action of synthetic cathinones at the CNS is presented in figure 3.

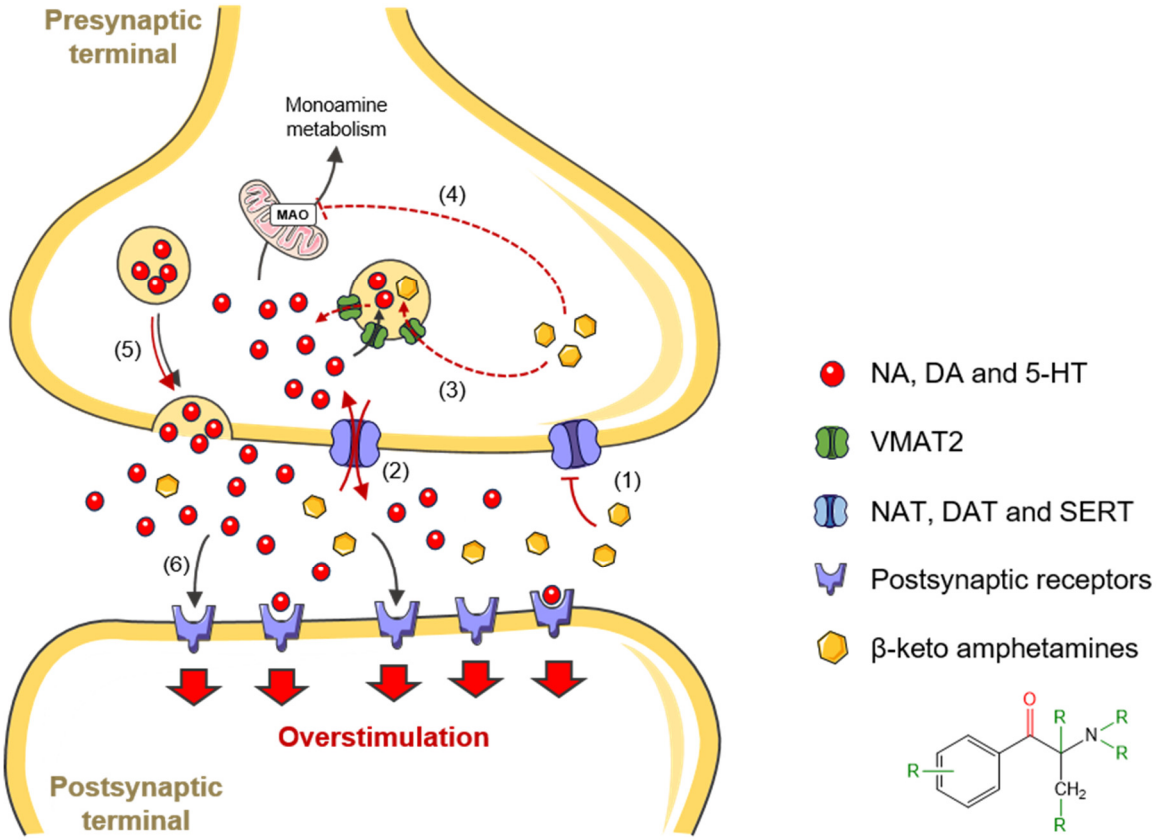

Figure 3: Putative pharmacological modes of action of β-keto amphetamines at the central nervous system. *β-keto amphetamines may act as blockers of NAT, DAT and SERT (1), inhibiting the uptake of catecholamines by the presynaptic terminals, and/or substrates (2), evoking transporter-mediated release of monoamines via reversal of normal transporter flux. Synthetic cathinones also inhibit VMAT2-mediated vesicular uptake of neurotransmitters (3), though with much lower potency than amphetamines, leading to the increase of neurotransmitters in the cytoplasm either by competing for the uptake as substrates, or by reversing the normal transporter flux. Evidence suggest the β-keto amphetamines may also inhibit MAO (4), the enzyme responsible for the inactivation of monoamines, which results in diminished neurotransmitter degradation. Both inhibitory mechanisms may further increase the availability of unsequestered biogenic amines to be released into the synaptic cleft (5). Catecholamines accumulated in the synaptic cleft bind to postsynaptic receptors (6), which results in overstimulation of postsynaptic cells and sympathetic activation. DAT – dopamine transporter; NET – norepinephrine transporter; SERT – serotonin transporter; MAO – monoamine oxidase; VMAT2 – vesicular membrane transporter 2.*

Unsequestered catecholamines within the synaptic terminal may undergo oxidative metabolism or autoxidation, with formation of highly reactive products that may induce oxidative stress, amplifying the toxicity of stimulant drugs (Cashman et al. 1999; Eisenhofer et al. 2004). Biogenic amines are metabolized intraneurally through several pathways. 5-HT is metabolized in two steps: first it undergoes oxidative deamination by MAO, yielding 5-hydroxyindole-3-acetaldehyde, which is further oxidized by aldehyde dehydrogenase into the end-product 5-hydroxyindoleacetic acid (Lam et al. 2010). Oxidative deamination of the

catecholamines DA and NE results in the formation of the biogenic aldehydes 3,4-dihydroxyphenylacetaldehyde (DOPAL) and 3,4-dihydroxyphenylglycolaldehyde (DOPEGAL), respectively. DOPAL and DOPEGAL can be further metabolized either through reduction by aldehyde reductase, oxidation by aldehyde dehydrogenase or *O*-methylation by catechol *O*-methyltransferase (COMT), with the main end-products being the homovanillic and vanillylmandelic acid, respectively (Eisenhofer et al. 2004). Growing evidence supporting the neurotoxic potential of endogenous DOPAL and DOPEGAL have emerged from the late 90's (Burke et al. 2003; Burke et al. 2001; Kristal et al. 2001; Legros et al. 2004; Mattammal et al. 1995), and several mechanisms have been proposed for the observed toxicity, including protein adduction, isoquinoline formation, production of free radicals and reactive oxygen species (ROS), and triggering of mechanisms that lead to apoptotic cell death (Marchitti et al. 2007).

Additionally to the mentioned catabolic pathways of monoamines, non-enzymatic oxidation may also take place, and the blocking of metabolism by MAO inhibitors, such as amphetamine and probably cathinone derivatives, may contribute to the rise of catecholamines autoxidation (Hurtado-Guzman et al. 2002). During this process, highly reactive *ortho*-quinones, hydroquinones, semiquinones and the aminochrome end-products are formed, which can further increase the neurotoxicity of psychostimulant drugs through increased production of ROS during redox cycling and oxidative stress, formation of covalent adducts with sulfhydryl groups with consequent depletion of glutathione (GSH) and enzymatic inactivation, as well as energy depletion due to uncoupling of ATP synthesis (Bindoli et al. 1992; Bindoli et al. 1989; Delcambre et al. 2016; Graham 1978).

The altered release of neurotransmitters may also play a role in β-keto amphetamines-elicited hepatotoxicity. The activation of α-adrenergic receptors located in the membrane of hepatocytes by endogenous catecholamines released due to cathinones action on membrane transporters can result in hepatic microvascular vasoconstriction and consequent compromise of liver perfusion, decreased hepatic GSH content, rise in cytosolic levels of free calcium and increased mitochondrial oxidation (Gonzalez-Manchon et al. 1988; Randle et al. 2008; Roberts et al. 1997; Taylor et al. 1983). Of note, evidence show that several cathinone derivatives, including 4-MMC and methcathinone, also have some binding affinity towards α-adrenergic receptors (Simmler et al. 2013a; Simmler et al. 2014), in particular α1-adrenoceptor, which is the predominant receptor in liver cells (Kulcsar-Gergely and Kulcsar 1989).

Lipophilicity and blood-to-brain dysfunction

The ability to cross cellular membranes may also influence the cytotoxic potential of drugs of abuse by modulating the amount that actually reaches the target cells. The neurotoxicity of psychostimulant substances is particularly co-related with the blood-brain barrier (BBB) permeability (Goncalves et al. 2014; Sharma et al. 2007; Yamamoto et al. 2010). Amphetamine derivatives have been shown to induce BBB dysfunction (Bowyer et al. 2008; Kiyatkin and Sharma 2009; Yamamoto and Bankson 2005). Although the mechanisms underlying the amphetamines-induced BBB damage have not been fully elucidated, evidence support a contributive role from drug-induced hyperthermia and oxidative stress (Ramirez et al. 2009; Sajja et al. 2016; Sharma et al. 2007). Considering the ability of β-keto amphetamines to trigger neuronal oxidative stress (Budzynska et al. 2015; Lopez-Arnau et al. 2015) and to raise the body temperature (Valente et al. 2014), the compromise of the BBB functions following use of cathinones might be anticipated.

Overall, the psychostimulant and pharmacological effects of β-keto amphetamines are less potent than their amphetamine analogues. For instance, the IC_{50} values of methylone-induced release of NA, DA and 5-HT are over 2.5-fold higher than MDMA in rat brain synaptosomes (Baumann et al. 2012; Baumann et al. 2013a). For this reason, to attain equipotent effects, the recreational doses reported by methylone users are usually higher than those of MDMA (Simmler et al. 2013a). The lower effects of synthetic cathinones may also underlie the need for redosing, which is often seen with 4-MMC (Deluca et al. 2009b; Schifano et al. 2011).

The presence of the β-keto group is known to increase the polarity of *N*-alkylated synthetic cathinone in comparison to their non-keto congeners, leading to a decline in their permeability and ability to cross the BBB and general bilipid membranes (Coppola and Mondola 2012a; Simmler et al. 2013a; Valente et al. 2014). In contrast, pyrovalerone derivatives have been suggested to easily cross the BBB due to their higher lipophilicity, which is conferred by the pyrrolidine ring-forming tertiary amine (Katz et al. 2014; Zaitsu et al. 2014). When comparing the BBB permeability of the most common cathinone and amphetamine derivatives in human brain cells expressing BBB transporters, Simmler et al. (2013a) found that MDPV, the compound with higher predicted lipophilicity, has the higher blood-to-brain and brain-to-blood permeability ratios. The lipophilic nature of MDPV enables extensive CNS stimulation, which helps explain the short onset of psychostimulant effects described by users for doses as low as 3 to 5 mg (Deluca et al. 2009a; Ross et al. 2012). Due to the significant difference found between apical-basolateral and basolateral-apical transport, active transport of MDPV across the BBB was proposed (Simmler et al. 2013a). In fact, MDPV was found to act as a substrate for a cocaine transporter present in the BBB,

and this carrier-mediated cocaine transport was demonstrated to be quantitatively much more important than its passive diffusion across the BBB (Chapy et al. 2014).

Notwithstanding the obvious similarities with amphetamine derivatives and the likelihood of β-keto amphetamines to compromise the BBB, the putative mechanisms of induced dysfunction still require clarification.

Metabolic bioactivation

Studies on the metabolism of synthetic cathinones have shown that these substances undergo a series of phase I pathways, including reduction of the ketone group with formation of an alcohol, *N*-dealkylation, hydroxylation of the carbonyl chain, oxidation of aromatic and *N*-alkyl groups, and opening of the pyrrolidine and methylenedioxy rings (for detailed information, please refer to Valente et al. 2014).

The pharmacokinetics of amphetamines has been thoroughly reviewed over the years (Carvalho et al. 2012; de la Torre et al. 2004a; de la Torre et al. 2004b; Lyles and Cadet 2003), and the metabolic activation of MDMA in particular plays a key role in both hepato- and neurotoxic potential of this substance (Capela et al. 2009; Capela et al. 2007; Carvalho et al. 2012; Carvalho et al. 2004a; Carvalho et al. 2010; Carvalho et al. 2004b; Lyles and Cadet 2003; Monks et al. 2004). MDMA presents two major pathways of hepatic metabolism, both mediated by cytochrome P450 (CYP450) isoenzymes, namely CYP2B6-mediated *N*-demethylation with formation of 3,4-methylenedioxyamphetamine (MDA), and CYP2D6-mediated demethylenation of both MDMA and MDA, yielding the catechol metabolites *N*-methyl-α-methyldopamine (*N*-Me-α-MeDA) and α-methyldopamine (α-MeDA) (Carvalho et al. 2012). Once formed in the liver, *N*-Me-α-MeDA and α-MeDA may enter the circulation and reach other organs, including the brain, where they exert their toxicity following further autoxidation with formation of highly reactive *ortho*-quinone intermediates, which may enter redox cycling with semiquinones with production of ROS and reactive nitrogen species (RNS), be further oxidized into toxic aminochromes like biogenic amines, or form GSH conjugates (Bindoli et al. 1989; Carvalho et al. 2012; Monks et al. 2004). Of note, MDMA-derived GSH conjugates proved to be more toxic than the parent compound (Barbosa et al. 2014; Capela et al. 2007; Carvalho et al. 2002b; Shenouda et al. 2009).

Like MDMA, *in vitro* studies, typically conducted in human liver microsomes, suggest that *O*-demethylenation is a major metabolic pathway for methylenedioxy cathinone derivatives, including methylone and MDPV, being catalyzed mainly by CYP2C19, 2D6 and 1A2 (Meyer et al. 2010a; Mueller and Rentsch 2012; Negreira et al. 2015; Pedersen et al. 2013; Springer et al. 2005; Strano-Rossi et al. 2010). From this reaction are formed

catechols that are further metabolized via COMT, yielding methylcatechols, which may be conjugated to form the phase II glucuronidated and sulphated metabolites (Negreira et al. 2015; Pedersen et al. 2013; Strano-Rossi et al. 2010). The analysis of urine specimens from β-keto amphetamines abusers and treated rats further confirmed the formation of catechols, methylcatechols and glucuronic acid conjugates (Bertol et al. 2014; Kamata et al. 2006; Meyer et al. 2010b). Of note, Meyer et al. (2014) showed the formation of GSH conjugates of five methylenedioxy cathinones, postulating conjugation at the aromatic ring. Despite the similarities to MDMA, there is a lack of evidence supporting the systemic formation of *ortho*-quinones from the metabolic activation of β-keto amphetamines. Nonetheless, a recent study by Wojcieszak et al. (2016) showed that the major metabolite of MDPV, catechol-MDPV, was significantly more toxic than the parent compound towards neuronal, hepatic and upper airway epithelium human cell lines, suggesting a major contribution of the catechol-metabolites to the neuro- and hepatotoxic effects of synthetic cathinones.

Neuroinflammation and hyperthermia

Neuroinflammation involving specific inflammatory factors and/or neuro-glial-microglia pathways has been theorized as a neurotoxic mechanism of β-keto amphetamines (Blum et al. 2013). Evidences show that MDMA- and METH-induced neuroinflammation involves activation of glial cells, which in turn leads to the release of pro-inflammatory factors and neuronal damage (Moratalla et al. 2015). Data on the neuroinflammatory effects of synthetic cathinones are scarce and somewhat inconsistent. For instance, Angoa-Perez et al. (2012) and Martinez-Clemente et al. (2014) found no signs of striatal or cortical astroglia or microglia activation in mice treated with 4-MMC in a binge-like regimen, but some astrocyte reactivity was observed in the hippocampus. Similar effects were observed in methylone-treated mice (Lopez-Arnau et al. 2014a), whereas only some cortical astrocyte reactivity was detected in adolescent rats treated with this cathinone (Lopez-Arnau et al. 2014b). In contrast, neither 4-MMC alone, nor the combination with either METH or MDMA induced significant astroglial or microglial activation in mice hippocampus (Angoa-Perez et al. 2014). Methcathinone induced a significant increase in glial fibrillary acid protein (GFAP), the marker of astroglial activation, in mice striatum (Anneken et al. 2017). Interestingly, though neither methylone nor MDPV alone prompted astroglial activation in this model, methylone significantly potentiated METH-induced increase in GFAP density, while MDPV had the opposite effect on amphetamine, METH and MDMA-induced astrogliosis, reducing GFAP density to control levels (Anneken et al. 2015). Importantly, astrogliosis, just like microglia activation, exacerbates

neuroinflammation via cytokine production (Brambilla et al. 2009), and it has also been implicated in METH-induced neuroinflammation, particularly at high doses or chronic exposure (Friend and Keefe 2013; Narita et al. 2008; Sakoori and Murphy 2010; Zhang et al. 2015).

Heat stress is closely related to the activation of glial cells and triggering of neuroinflammatory effects (Auvin et al. 2009; Bahniwal et al. 2011; Lee et al. 2015), and brain hyperthermia is known to exacerbate the harmful neuronal effects of amphetamines (Bowyer et al. 1994; Brown and Kiyatkin 2004; Kiyatkin et al. 2007; Malberg and Seiden 1998). Sympathetic overstimulation, mediated predominantly α-adrenergic receptors, more importantly α_1-adrenoceptor, are known to contribute to the hyperthermic effect of amphetamines (Bexis and Docherty 2008; Kikuchi-Utsumi et al. 2013; Matsumoto et al. 2014). In the work of Simmler et al. (2013a), cathinone and the derivatives methcathinone, 4-MMC and flephedrone, exhibited *in vitro* affinity towards α_1-adrenoceptor. In humans, robust increases in body temperature were reported during acute intoxication with 4-MMC (Garrett and Sweeney 2010; Kasick et al. 2012; Lusthof et al. 2011; Zona et al. 2016), methylone (Barrios et al. 2016; Pearson et al. 2012; Warrick et al. 2012; Zona et al. 2016) and MDPV (Beck et al. 2015; Borek and Holstege 2012; Kesha et al. 2013; Mugele et al. 2012; Murray et al. 2012; Valsalan et al. 2017; Young et al.), and, as observed for MDMA (Hysek et al. 2012; Sprague et al. 2005), the α- and β-adrenergic antagonist carvedilol effectively blocked the hyperthermic effect of 'bath salts' containing methylone and 4-MMC (Zona et al. 2016). However, *in vivo* animal studies addressing the effects of these substances on body temperature are controversial, showing variations dependent on dose, species, sex and experimental conditions, especially ambient temperature. For instance, MDPV induces hyperthermia in adult male F344/N, LEW and Sprague-Dawley rats but not in adolescent male Sprague-Dawley rats (King et al. 2014; Merluzzi et al. 2014). However, Aarde et al. (2015) found a dose-dependent decrease in body temperature of MDPV- and α-PVP-treated male Wistar rats. Adam et al. (2014) showed that a 10 mg/kg dose of MDPV had no effect on the core body temperature of mixed sex C57BL/6j mice, while Anneken et al. (2015) found that 30 mg/kg of MDPV elicited a hyperthermia in female mice of the same species at normal ambient temperature. In contrast, the same dose was shown to induce hyperthermia on male NIH Swiss mice but only at warm ambient temperature (28ºC) (Fantegrossi et al. 2013; Gannon et al. 2016). Also in male NIH Swiss mice, MDPV-induced hyperthermic effects proved to be stereoselective, with the *S*(+) enantiomer being generally more potent than *R*(-)-MDPV (Gannon et al. 2016). Data on 4-MMC and methylone is more consistent, with a general dose-dependent production of hyperthermia in both rats and mice (Angoa-Perez et al. 2013; Anneken et al. 2015; Baumann et al. 2012; den Hollander et al. 2013; Kiyatkin et al. 2015; Lopez-Arnau et al. 2014a; Martinez-Clemente et al. 2014; Piao

et al. 2015), though some *in vivo* studies also show transient or sustained hypothermic responses following 4-MMC administration, as well as a dependence of ambient temperature and species in the thermoregulation by this derivative (den Hollander et al. 2013; Lopez-Arnau et al. 2015; Miller et al. 2013; Shortall et al. 2013; Shortall et al. 2016) Of note, Kiyatkin et al. (2015) showed that both methylone and MDPV trigger *in vivo* dose-dependent brain hyperthermia accompanied by peripheral vasoconstriction, which appears to be a critical mechanism underlying MDMA-induced neurodamage (Kiyatkin et al. 2014).

Drug-induced hyperthermia was also related to the hepatotoxic potential of amphetamines (Carvalho et al. 2010; Matsumoto et al. 2014), and *in vitro* data further confirmed that the rise in temperature exacerbates the hepatotoxic effects of MDMA, METH and amphetamine (Carvalho et al. 1997; Carvalho et al. 2001b; Carvalho et al. 2002a; Dias da Silva et al. 2014; Halpin et al. 2013; Pontes et al. 2008a).

Of note, the rise in body temperature *per se* is an aggressively deleterious condition to the liver and brain (Ando et al. 1994; Kiyatkin 2005; Santos-Marques et al. 2006; Sharma 2006; Skibba et al. 1991), and hyperthermia is recognized as one of the most life-threatening effects of amphetamines intoxications, leading to serious or even fatal complications such as rhabdomyolysis, disseminated intravascular coagulation and multiorgan system failure (Carvalho et al. 2012). Considering the thermoregulatory properties of β-keto amphetamines, further studies regarding the influence of hyperthermia on the toxic effects of these substances are essential for a comprehensive treatment approach of ‘bath salt’-related intoxications.

Mitochondrial dysfunction and programmed cell death

Mitochondria play vital roles in the maintenance of cell survival: these organelles are the main suppliers of energy in eukaryotic cells, generating adenosine triphosphate (ATP) through fatty acid β-oxidation and mainly oxidative phosphorylation (Nsiah-Sefaa and McKenzie 2016); they are a major source of ROS and RNS, which appear to act not just as harmful by-products of mitochondrial respiration but also as cell signaling modulators (Brookes et al. 2002); they are involved in programmed cell death through the opening of the mitochondrial permeability transition pore, release of cytochrome *c*, and subsequent initiation of mitochondria-dependent apoptotic pathway (Wang and Youle 2009). For all these reasons, mitochondria are primary targets of drugs of abuse and mitochondrial dysfunction is a central mechanism of drug-induced toxicity in several organs, including the liver and the brain (Dykens and Will 2008).

The key role of mitochondria in the hepatic and neuronal toxicity of amphetamines has been thoroughly reviewed over the years, and appear to involve inactivation of key

mitochondrial enzymes, inhibition of mitochondrial electron transport chain's complexes, uncoupling of oxidative phosphorylation, oxidative modifications in mitochondrial macromolecules, and upstream initiation of apoptotic cell death (Barbosa et al. 2015; Brown and Yamamoto 2003; Cadet et al. 2007; Carvalho et al. 2010).

Thus far, information on β-keto amphetamines-induced mitochondrial dysfunction is scarce. *In vitro* evidences suggest that cathinone derivatives elicit a time- and concentration-dependent decline in mitochondrial activity, as demonstrated by Wojcieszak et al. (2016) in neuronal, hepatic and upper airway epithelium cells exposed to α-pyrrolidinovalerothiophenone, α-pyrrolidinooctanophenone, MDPV and its catechol and methylcatechol metabolites. 4-MMC was shown to reduce the basal and induced maximal mitochondrial respiration in neuronal cells, producing a significantly larger decrease than METH (den Hollander et al. 2014). Interestingly, den Hollander et al. (2015) showed that the effect of cathinones on the mitochondrial respiration may be related to the formation of membrane insoluble break down products (methylbenzamides), with the methylbenzamides formed from methylone and 4-MMC inducing significant decrease in the basal respiration at conditions where the parent compounds had little to no effects. Methylone also elicited a significant concentration-dependent ATP depletion in isolated rat hepatocytes, which was followed by membrane blebbing and cell death, and a significant dissipation of the mitochondrial membrane potential (Δψm) (Nakagawa et al. 2009). The ability to induce apoptotic cell death was demonstrated for MDPV in neuronal cells, with a concentration-dependent rise in DNA fragmentation (Rosas-Hernandez et al. 2016a), and in several regions of neonatal mouse brain, with significant increase in caspase 3 activation (Adam et al. 2014). More recently, Matsunaga et al. (2017) assessed the ability of several α-pyrrolidinophenone derivatives to induce apoptosis in human aortic epithelial cells, showing a general increase in caspase 3 activation after 48 h of exposure. Furthermore, the neuronal cell death induced by 4-MMC was significantly reversed in cells overexpressing the anti-apoptotic B-cell lymphoma 2 (Bcl-2) gene, supporting the contribution of apoptotic cell death to the neurotoxicity of this cathinone derivative (den Hollander et al. 2015).

In addition to apoptosis, autophagy is another programmed cell death pathway by which cells dispose of damaged organelles and non-functional proteins, and that appears to be upregulated in response to stress or a toxic insult, ultimately leading to cell death, either by cellular atrophy and collapse of cellular functions (autophagic cell death), or through the activation of apoptotic or necrotic cell death (Galluzzi et al. 2008).

The increase in autophagic activation triggered by METH and MDMA has been related to their neurotoxic effects, although the role of autophagy in the observed neurodegeneration remains a debatable matter, with evidences supporting the neuroprotective role of autophagy (Castino et al. 2008; Lin et al. 2012; Mercer et al. 2017;

Pitaksalee et al. 2015), as well as the potentiation of neurotoxicity due to instigation of autophagic cell death (Li et al. 2016; Li et al. 2014). However, there are currently no studies addressing the ability of β-keto amphetamines to trigger autophagy, or its involvement in either neurotoxic or hepatotoxic effects of these substances.

Oxidative stress

Recent studies support the ability of β-keto amphetamines to trigger oxidative stress in different cell types, including neuronal cells, characterized by an overall increase in ROS and RNS production (Matsunaga et al. 2017; Rosas-Hernandez et al. 2016a; Rosas-Hernandez et al. 2016b). However, the mechanisms underlying the elicited oxidative stress are not fully elucidated.

Most of the previously described putative cellular mechanisms of hepato- and neurotoxicity of β-keto amphetamines may lead to an increase in intracellular oxidative stress, in comparable ways to amphetamines (Carvalho et al. 2012).

For instance, catecholamine metabolism and non-enzymatic autoxidation lead to the production of highly reactive metabolites, as well as ROS and RNS that are capable of initiating oxidative damage (Costa et al. 2011; Eisenhofer et al. 2004; Miller et al. 1996).

As aforementioned, a MDMA-like metabolic bioactivation may be hypothesized for methylenedioxy β-keto amphetamines, such as methylone and MDPV, with oxidation of their catechol metabolites, which were already validated both *in vitro* and *in vivo* (Kamata et al. 2006; Meyer et al. 2010a; Pedersen et al. 2013; Strano-Rossi et al. 2010), and formation of unstable *ortho*-quinones that enter redox cycling with their *ortho*-semiquinone radicals with consequent production of ROS and RNS (Capela et al. 2006).

Microglial activation is known to underlie the neuroinflammatory effects of amphetamines (Carvalho et al. 2012; Halpin et al. 2014), but it also mediates the release of reactive species, including $O_2^{\cdot-}$ and nitric oxide (NO), that potentiate neuronal oxidative damage (Thomas et al. 2004). β-Keto amphetamines, on the other hand, appear to have little to no effect on microglia, but evidences suggest that they may trigger astrocyte activation (Anneken et al. 2017; Lopez-Arnau et al. 2014a; Lopez-Arnau et al. 2014b; Martinez-Clemente et al. 2014). Astrocytes are the most numerous and diverse specialized glial cells in the CNS, playing several roles in the preservation of CNS health, including the regulation of local blood flow and the maintenance of transmitter homeostasis required for a healthy synaptic transmission (Sofroniew and Vinters 2010). Among the list of detrimental effects following excessive astrocyte activation, also referred as reactive astrogliosis, stands out the generation of neurotoxic levels of reactive species, in particular NO, as a

result of the upregulation of the inducible form of NO synthase, contributing to delayed neuronal death (Swanson et al. 2004).

Hyperthermia is also known to exacerbate the oxidative stress triggered by amphetamines in both liver and brain models (Barbosa et al. 2014; Carvalho et al. 2001b; Pontes et al. 2008b). As aforementioned, hyperthermia is a common complication following exposure to cathinones (Borek and Holstege 2012; Pearson et al. 2012) and, despite the lack of *in vitro* experimental evidences regarding the potential effects of the rise in body temperature on β-keto amphetamines-induced neuro- and hepatotoxicity, *in vivo* studies show that the effects of MDPV on locomotor stimulation are significantly affected by ambient temperature (Fantegrossi et al. 2013; Gannon et al. 2016). Importantly, hyperthermia *per se* triggers a prooxidant state, characterized by the depletion of the cellular antioxidant GSH and consequent rise in the oxidized form, glutathione disulfide (GSSG), potentiation of lipid peroxidation, and increased conversion of xanthine oxidase through proteolysis from the physiological type-D to type-O form, which generates $O_2^{\cdot -}$ and H_2O_2 by the reduction of oxygen during oxidation of xanthine or hypoxanthine (Kiyatkin 2005; Santos-Marques et al. 2006; Sharma 2006; Skibba et al. 1991).

There is a complex cellular crosstalk involving intracellular Ca^{2+}, the mitochondria and ROS, in which the ROS generated as a result of an oxidative insult elicits Ca^{2+} overload in the mitochondria, triggering the stimulation of NO synthase and consequent generation of NO, which in turn inhibits the complex IV of the electron transport, leading to mitochondrial dysfunction and production of more ROS at complex III, thus propagating the oxidative damage (Brookes et al. 2004). The work of Votyakova and Reynolds (2001) showed that the majority of the ROS generation in rat brain mitochondria results from the inhibition of the electron transport at complex I, a mode of action that is also responsive to depolarization of the Δψm. For this reason, substances that interfere with mitochondrial respiration, either by increasing the Ca^{2+} levels, inhibiting the mitochondrial electron transport or disrupting the Δψm, may effectively lead to mitochondrial ROS generation. Of note, Nakagawa et al. (2009) showed that methylone significantly disrupts the Δψm in rat hepatocytes, leading to a depletion of ATP levels. However, there are few studies addressing the effects of synthetic cathinones on mitochondrial functions, and besides the suggestion of decreased mitochondrial activity in treated cells (den Hollander et al. 2014; den Hollander et al. 2015; Wojcieszak et al. 2016), little is known about the mechanisms underlying these effects or their relevance in mitochondrial ROS production and oxidative stress in general.

An antioxidant deficiency can also result in oxidative stress (Davies 2000). There is mounting evidence that amphetamines trigger depletion of the antioxidant defense systems, which includes the drop of cellular GSH levels (Carvalho et al. 2002a; Pontes et al. 2008a),

downregulation of the antioxidant family of peroxiredoxins and upregulation of peroxiredoxin reducing systems (Chen et al. 2007), and disturbance in the activity and/or expression of several antioxidant enzymes such as catalase (Zhou et al. 2003), Cu-Zn superoxide dismutase (El-Tawil et al. 2011; Jayanthi et al. 1999), glutathione reductase (Carvalho et al. 2001a), glutathione peroxidase (Carvalho et al. 2002a; Jayanthi et al. 1998), and glutathione *S*-transferase (Pontes et al. 2008a). Accordingly, 4-MMC was also shown to induce oxidative stress in the brain of rats and mice, characterized by increased lipid peroxidation and variations in the expression of catalase, NO synthase, superoxide dismutase, and glutathione peroxidase (Budzynska et al. 2015; Ciudad-Roberts et al. 2016; Lopez-Arnau et al. 2015).

In summary, aside from the pharmacological mechanisms of action of β-keto amphetamines, current knowledge on the cytotoxic mechanisms underlying the hepatotoxic and neurotoxic effects of these novel drugs of abuse is still scarce and requires further assessment.

Section II – Objectives

Considering the prominent rise of NPS abuse worldwide, the information presented in the general introduction regarding the potential for adverse effects from β-keto amphetamines use, and the scarcity of experimental data on their cellular toxicity, the necessity of conducting a study addressing the mechanisms underlying the toxic effects of these psychostimulant substances became apparent.

Of particular concern is the target-organ toxicity of β-keto amphetamines, namely the hepato- and neurotoxic effects, as clinical evidence suggests that both the liver and the CNS are major targets of these NPS.

β-Keto amphetamines share many resemblances to classical amphetamines, particularly with regards to chemical structure and pharmacological effects, and, thus, similar toxicological effects may also be anticipated.

Hence, the present thesis aimed at improving the current knowledge on the cellular mechanisms underlying the toxicity of β-keto amphetamines by means of *in vitro* cell models suitable for the assessment of the cellular hepatic and neuronal effects of these prominent recreational drugs, hopefully contributing for the development of a comprehensive treatment approach of β-keto amphetamine-related intoxications.

The specific purposes of this thesis were:

1. To clarify the mechanisms underlying the hepatotoxicity of several commonly abused β-keto amphetamine derivatives, including methylone, MDPV, pentedrone and 4-MEC, using primary cultured rat hepatocytes and the human hepatic cell line HepaRG, and compare them to the well-known hepatotoxic amphetamine derivative, MDMA;
2. To assess the effects of hyperthermia on the observed hepatotoxicity of β-keto amphetamines by comparison of effects on primary cultured rat hepatocytes under normo- and hyperthermic conditions exposed to MDPV, a derivative with documented clinical hyperthermic response;
3. To elucidate the cellular neurotoxic effects of methylone and MDPV in a comparative study to MDMA, using undifferentiated and dopaminergic neuronal SH-SY5Y cells, with special focus on oxidative stress, mitochondrial dysfunction and cell death mechanisms;
4. To determine the role of autophagy in β-keto amphetamines-induced neurotoxicity and its relationship with oxidative stress and apoptosis.

Section III – Original research

This section presents a thorough description of the materials and experimental methods, the obtained results and their discussion within each experimental study, in the form of peer-reviewed publications or submitted manuscript. The following studies are included:

Manuscript I. Valente MJ, Araújo AM, Bastos ML, Fernandes E, Carvalho F, Guedes de Pinho P, Carvalho M (2016) Characterization of hepatotoxicity mechanisms triggered by designer cathinone drugs (β-keto amphetamines). *Toxicol Sci* 153(1):89-102 doi:10.1093/toxsci/kfw105

Manuscript II. Valente MJ, Araújo AM, Silva R, Bastos ML, Carvalho F, Guedes de Pinho P, Carvalho M (2016) 3,4-Methylenedioxypyrovalerone (MDPV): *in vitro* mechanisms of hepatotoxicity under normothermic and hyperthermic conditions. *Arch Toxicol* 90(8):1959-73 doi:10.1007/s00204-015-1653-z

Manuscript III. Valente MJ, Bastos ML, Fernandes E, Carvalho F, Guedes de Pinho P, Carvalho M (2017) Neurotoxicity of β-keto amphetamines: deathly mechanisms elicited by methylone and MDPV in human dopaminergic SH-SY5Y cells. *ACS Chem Neurosci* doi:10.1021/acschemneuro.6b00421

Manuscript IV. Valente MJ, Amaral C, Correia-da-Silva G, Duarte JA, Bastos ML, Carvalho F, Guedes de Pinho P, Carvalho M (2017) Methylone and MDPV activate autophagy in human dopaminergic SH-SY5Y cells: a new insight into the context of β-keto amphetamines-related neurotoxicity. Submitted manuscript

Manuscript I. Characterization of hepatotoxicity mechanisms triggered by designer cathinone drugs (β-keto amphetamines)

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Characterization of Hepatotoxicity Mechanisms Triggered by Designer Cathinone Drugs (β-Keto Amphetamines)

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ABSTRACT

The use of cathinone designer drugs in recreational settings has been associated with severe toxic effects, including liver damage. The precise mechanisms by which cathinones induce hepatotoxicity and whether they act by common pathways remain to be elucidated. Herein, we assessed the toxicity of the cathinones methylone, pentedrone, 3,4-methylenedioxypyrovalerone (MDPV) and 4-methylethcathinone (4-MEC) in primary rat hepatocytes (PRH) and HepaRG cells, and compared with that of 3,4-methylenedioxymethamphetamine (MDMA). MDPV and pentedrone were significantly more toxic than MDMA, while methylone was the least cytotoxic compound. Importantly, PRH revealed to be the most sensitive experimental model and was thus used to explore the mechanisms underlying the observed toxicity. All drugs elicited the formation of reactive oxygen and nitrogen species (ROS and RNS), but more markedly for methylone, pentedrone and 4-MEC. GSH depletion was also a common effect at the highest concentration tested, whereas only MDPV and pentedrone caused a significant decrease in ATP levels. The antioxidants ascorbic acid or *N*-acetyl-L-cysteine partially attenuated the observed cell death. All cathinones triggered significant caspase activation and apoptosis, which was partially reversed by the caspase inhibitor Ac-LETD-CHO. In conclusion, the present data shows that (1) cathinones induce *in vitro* hepatotoxic effects that vary in magnitude among the different analogues, (2) oxidative stress and mitochondrial dysfunction play a role in cathinones-induced hepatic injury, and (3) apoptosis appears to be an important pathway of cell death elicited by these novel drugs.

Key words: synthetic cathinones; β-keto amphetamines; hepatotoxicity; oxidative stress; apoptosis.

In the past few decades, classical illicit drugs have been gradually losing ground to new psychoactive substances (NPS), especially among young adult users in recreational settings (Burns *et al.*, 2014; Helander *et al.*, 2014; Maxwell, 2014). Synthetic cathinones, often deceivingly labelled as 'bath salts', are a major family of NPS that encompasses a large number of psychoactive substances deliberately designed to mimic the effects of controlled drugs of abuse (Karila *et al.*, 2015). According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), synthetic cathinones were the largest category of NPS identified in Europe in 2014, with 31 new derivatives reported for the first time that year (EMCDDA, 2015a). 3,4-Methylenedioxymethcathinone (methylone), 3,4-methylenedioxypyrovalerone (MDPV), α-methylaminovalerophenone (pentedrone) and 4-methylethcathinone (4-MEC) are four of the most commonly used derivatives worldwide (Elliott and Evans,

2014; Moran and Seely, 2014). Together, these four derivatives covered over 40% of all samples of synthetic cathinones seized in Europe in 2013 (EMCDDA, 2015b), and were in the top five of most consumed synthetic cathinones in the United States in the same year (DEA, U.S 2014). Along with the paradigm shift in recreational drug use, several reports of intoxication and deaths related to the use of designer cathinone drugs have been described in Maskell *et al.* (2011), Regunath *et al.* (2012), Ross *et al.* (2011), and in particular with these 4 derivatives (Gil *et al.*, 2013; Murray *et al.*, 2012; Pearson *et al.*, 2012; Sykutera *et al.*, 2015), raising a global health concern over the abuse of these NPS.

Chemically, synthetic cathinones are related to amphetamines, bearing a ketone group at the β-position of the side chain, and are therefore often named β-keto amphetamines (Prosser and Nelson, 2012). Functional group substitutions to the core structure of the parent cathinone compound have yielded a large number of NPS on the street and cyber drug markets (EMCDDA, 2015b), which can be separated into different chemical families based on the substitutions made (Valente *et al.*, 2014). The most basic derivatives are the *N*-alkylated derivatives, which may present alkyl substitutions in the α-carbon of the side chain and in the benzyl ring. When a 3,4-methylenedioxy group is added to the benzyl ring, cathinones structurally similar to 3,4-methylenedioxyamphetamines are produced (Kelly, 2011). The pyrrolidinophenone-like family encompasses derivatives containing a pyrrolidine substitution in the nitrogen atom (Westphal *et al.*, 2007), which can be further modified to combine the pyrrolidine moiety and a 3,4-methylenedioxy ring (Kelly, 2011), yielding more complex cathinones. Importantly, the apparent boom in NPS creates a new challenge from a chemical and biological point of view, as structures, solubility and redox potential of the designer drugs differ between NPS and the prototype drugs they are derived from (den Hollander *et al.*, 2014). Generally, the presence of the β-keto group increases the polarity of cathinone derivatives, resulting in a decrease of their ability to cross lipid bilayer membranes, such as the blood-brain barrier (BBB) (Simmler *et al.*, 2013, 2014), which consequently decreases the potency of synthetic cathinones when compared with their related amphetamines. For this reason, users often resort to higher doses or binge consumption of cathinone derivatives to attain equipotent effects (Prosser and Nelson, 2012). The polarity issue occurs mainly with the *N*-alkylated derivatives, but not so much with the pyrrolidine family of cathinones, since the presence of the pyrrolidine ring greatly reduces the polarity of these compounds (Coppola and Mondola, 2012), resulting in a greater transport of derivatives such as MDPV through the BBB.

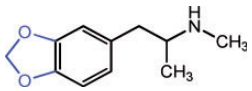
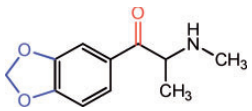
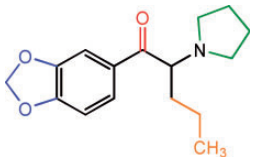
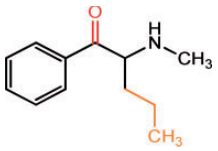
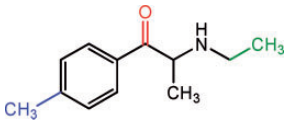
Designer cathinone drugs exert their stimulant effects by interacting with monoamine membrane transporters, namely dopamine (DA), norepinephrine (NE), and serotonin (5-HT) transporters (Baumann *et al.*, 2012; Cozzi *et al.*, 1999; Eshleman *et al.*, 2013; Kelly, 2011; Lopez-Arnau *et al.*, 2012). However, considerable differences have been found in the affinities towards these transporters among the different substituted cathinones *in vitro*. Methylone, like amphetamines (Sitte and Freissmuth, 2010), acts as a substrate for monoamine transporters to induce reverse transport of neurotransmitters, which consequently leads to increased concentration of these biogenic amines in the synaptic cleft, but with weaker potency than MDMA (Baumann *et al.*, 2012). Conversely, MDPV and pentedrone function as pure transporter blockers, resembling cocaine, inhibiting the uptake of DA and NE, with minimal effects on 5-HT uptake (Baumann *et al.*, 2013; Simmler, *et al.*, 2014). 4-MEC presents a mixed mode of interaction, with a potency of inhibition similar to cocaine, but also a 5-HT release comparable to MDMA (Simmler, *et al.*, 2014). As a more dopaminergic and noradrenergic drug, MDPV induces MDMA- and cocaine-like subjective effects, including increased energy, but limited euphoria and only mild empathogenic effects (Deluca *et al.*, 2009), while methylone, 4-MEC and pentedrone users report more MDMA-like stimulating effects, such as euphoria, openness and increased sociability and sexual drive (Van Hout, 2014; www.drugs-forum.com). Whereas variations on the mode of action of synthetic cathinones appear to be determined by their chemical structure (Simmler, *et al.*, 2014), it remains to be studied whether the mechanisms of toxicity elicited by this group of NPS are also reliant on their structure. Common symptoms of 'bath-salts'-induced intoxications are consistent with sympathetic stimulation, including hypertension, hyperthermia, tachycardia and seizures, and often result in end-organ effects and death (Coppola and Mondola, 2012; Prosser and Nelson, 2012). Since ingestion is one of the preferable routes of administration for this group of NPS (Valente *et al.*, 2014), the first-pass effect renders the liver more susceptible to toxic injury, as ascertained by clinical evidences of cathinones-induced hepatic dysfunction (Borek and Holstege, 2012; Carbone *et al.*, 2013; Fröhlich *et al.*, 2011; Murray, *et al.*, 2012; Pearson, *et al.*, 2012) and organ accumulation (Marinetti and Antonides, 2013; Sykutera, *et al.*, 2015). Therefore, the aim of this study was to investigate *in vitro* the effects and mechanisms underlying the hepatotoxicity of four designer cathinone drugs belonging to different chemical families. The structures and some physicochemical properties, namely the partition coefficient (log *P*) and the acid dissociation constant (pK_a), of cathinones herein studied are presented in Table 1. Methylone, which belongs to the group containing a 3,4-methylenedioxy ring, only differs from MDMA by the presence of the β-keto group, thus earning the common designation of βk-MDMA. MDPV, from the 3,4-methylenedioxy-*N*-pyrrolidine family, is characterized by a side chain alkylation (butylation), which is also found in pentedrone. Pentedrone is included in the *N*-alkylated family, with a *N*-methyl group, as well as 4-MEC, which presents a methyl group in the benzyl ring, and a *N*-ethyl group. Considering the structural similarity of cathinone derivatives and MDMA, a comparative study was conducted. The specific objectives of this study were to (1) evaluate the cytotoxic potential of selected cathinones in primary rat hepatocytes (PRH) and HepaRG cells, and compare it to MDMA, (2) provide further chemical characterization by assessing the redox potential of cathinone derivatives, (3) elucidate the cellular mechanisms underlying the observed hepatotoxicity, specifically the induction of oxidative stress and the interference with mitochondrial function, and (4) characterize the cell death pathways involved in liver damage elicited by these NPS.

MATERIALS AND METHODS

Chemicals

Collagenase from *Clostridium histolyticum* Type IA, Williams' E medium, dexamethasone, gentamicin, insulin solution from bovine pancreas (10 mg/ml), hydrocortisone 21-hemisuccinate sodium salt, thiazolyl blue tetrazolium bromide (MTT), sodium pyruvate, β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (β-NADH), L-glutathione reduced (GSH) and L-glutathione oxidized disodium salt (GSSG), β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (β-NADPH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 2′,7′-dichlorodihydrofluorescein (DCFH) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), adenosine triphosphate (ATP), luciferase from *Photinus*

TABLE 1. Chemical Structures and Logarithmic Values of the Acid Dissociation Constant (pK_a) and the Partition Coefficient (log *P*) of the Studied Compounds

Common Name	Chemical Structures	pK_a*	Log *P**
MDMA (3,4-methylenedioxymethamphetamine)		10.14	1.92
Methylone (3,4-methylenedioxy-*N*-methylcathinone)		7.96	1.08
MDPV (3,4-MDPV)		7.31	2.63
Pentedrone (α-methylaminovalerophenone)		8.20	2.26
4-MEC (4-methylethcathinone)		8.13	2.21

*pKa and log *P* values were calculated using MarvinSketch v.16.2.29.0 software (ChemAxon, Budapest, Hungary).

pyralis (firefly) and D-luciferin sodium salt, *N*-acetyl-Asp-Glu-Val-Asp-*p*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide (Ac-IETD-pNA), and *N*-acetyl-Leu-Glu-His-Asp-*p*-nitroanilide (Ac-LEHD-pNA), *N*-acetyl-Leu-Glu-Thr-Asp-aldehyde (Ac-LETD-CHO), *N*-acetyl-L-cysteine (NAC), ascorbic acid (AA) and bovine serum albumin were purchased from Sigma Aldrich (St Louis, Missouri). Heat-inactivated fetal bovine serum (FBS), antibiotic mixture of penicillin/streptomycin (10,000 U/ml/10,000 μg/ml), fungizone (250 μg/ml), and Hank's balanced salt solution (HBSS) were obtained from GIBCO Invitrogen (Barcelona, Spain). All other chemicals of analytical grade were purchased from Merck (Darmstadt, Germany). Hydrochloride salts of methylone, MDPV, pentedrone and 4-MEC were purchased online from the Sensearomatic website (http://sensearomatic.net, currently unavailable), during March 2013. MDMA was extracted, purified, and converted to the respective hydrochloride salt at UCIBIO-REQUIMTE, Laboratory of Toxicology, Faculty of Pharmacy, Porto, Portugal, from high purity MDMA tablets provided by the Portuguese Criminal Police Department. The salts were fully characterized by mass spectrometry, NMR and elemental analysis (data not shown), and purity was >98%.

In Vitro *Hepatotoxicity Cellular Models*

HepaRG cell culture. HepaRG cell line was supplied by Life Technologies Europe BV (Bleiswijk, the Netherlands) and routinely maintained in complete Williams' E medium, supplemented with 10% FBS, 100 U/ml/100 μg/ml penicillin/streptomycin, 5 μg/ml insulin and 50 μM hydrocortisone 21-hemisuccinate, and incubated at 37°C, with 5% CO_2. Cells were subcultured over 6 passages (passages 9–14). When reaching confluence, 2% DMSO was added to the culture medium to allow cell differentiation hepatocyte-like cells into adult hepatocytes and biliary epithelial cells. After a 2-week differentiation period, cells were seeded in 96-well plates at high density (0.45×10^6 cells/cm^2), and incubated overnight at 37°C, with 5% CO_2, to allow cell adhesion.

Isolation and primary culture of rat hepatocytes. Male Wistar Han rats weighing 210–250 g were purchased from Charles-River Laboratories (Barcelona, Spain). Surgical procedures were conducted under isoflurane-induced anesthesia in an isolated system, and carried out between 10.00 and 11.00 am. All experiments were approved by the Ethics Committee of the Faculty of Pharmacy, University of Porto.

Isolated rat hepatocytes were obtained through a collagenase perfusion, as previously described in Valente *et al.* (forthcoming). Cell viability was assessed through the trypan blue exclusion test and was always higher than 80%. A suspension of 0.5×10^6 viable cells/ml was cultured in 6- or 96-well plates at approximately 0.1×10^6 cells/cm^2, in Williams' E medium, supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 50 μM dexamethasone, 100 μg/ml gentamicin, and 2.5 μg/ml fungizone, and incubated overnight at 37°C, with 5% CO_2, to allow cell adhesion.

Drug Treatments

Primary cultured hepatocytes and HepaRG cells were exposed for 24 h to the four cathinones — methylone, MDPV, pentedrone and 4-MEC — and MDMA, at a wide concentration range, from 0.05 to 10 or 20 mM, in order to obtain a complete concentration-response curve in the MTT reduction assay. All incubations with the test drugs were performed in serum-free medium. For further assays, performed only in PRH, the five compounds were tested at a narrower range that includes low-effect to worst-case approach concentrations (0.2–1.6 mM). To

evaluate the effect of cytochrome P450 2D6 isoenzyme (CYP2D6) inhibitor quinidine, the antioxidants AA and NAC, or the caspases inhibitor Ac-LETD-CHO, a pre-incubation of 1 h was performed, followed by a co-incubation with MDMA or the synthetic cathinones at 1.6 mM.

Characterization of the Reducing Properties of Synthetic Cathinones

The reducing power of cathinone derivatives and MDMA was determined as described by Berker *et al.* (2007). Briefly, various concentrations (0.05–50 mM) of each compound were mixed with 0.2 M sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide (w/v). The mixture was incubated at 50°C for 20 min. After incubation, 10% tricloroacetic acid (w/v) was added and the mixture was centrifuged for 8 min at 1000 rpm, at 4°C. The upper layer was mixed with equal volume of deionised water and 0.1% of ferric chloride (w/v). Depending on the reducing potential of each compound, the yellow color of the initial solution changes to various shades of green due to the formation of Prussian blue, $KFe[Fe(CN)_6]$, from the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, the formation of Prussian blue at 700 nm provides a measurement of the Fe^{2+} concentration in each solution. Results were obtained from three independent experiments, run in duplicate.

Cell Viability Assessment

MTT reduction and LDH leakage assays were performed as previously described (Valente *et al.*, forthcoming). Data were obtained from at least five independent experiments, performed in triplicate, and normalized to no treatment and 1% Triton X-100 controls.

Characterization of Hepatotoxicity Mechanisms Triggered by Cathinone Derivatives

Intracellular ROS and RNS formation. Intracellular production of reactive oxygen (ROS) and nitrogen (RNS) species was evaluated by the inclusion of the probe DCFH-DA (Valente *et al.*, forthcoming). As determined by incubation of MDMA or cathinone derivatives for 24 h with DCFH, in the absence of cells, no interference with the probe was noted at any tested concentration (data not shown). Results were obtained from six independent experiments, performed in triplicate, and normalized to cells with no treatment.

Intracellular GSH and GSSG levels. Total and oxidized glutathione (GSSG) intracellular contents were determined by the DTNB-GSSG reductase recycling assay (Valente *et al.*, forthcoming). GSH concentrations were calculated as follows: [GSH] = [total glutathione] – 2*[GSSG]. GSH and GSSG levels were normalized to total protein contents, as determined through the Lowry method. Results were obtained from at least four independent experiments, run in duplicate.

Intracellular ATP levels. Intracellular levels of ATP were quantified through a bioluminescence assay based on the emission of light from the reaction of ATP and luciferin, catalyzed by the enzyme luciferase (Valente *et al.*, forthcoming). No interference with the probe was noted at any tested concentration, as determined by measurement of the bioluminescence of MDMA or cathinone derivatives solutions with luciferin-luciferase assay solution in the absence of cells (data not shown). Results were obtained from at least four independent experiments, run in duplicate. ATP contents were normalized to total protein.

Characterization of Cell Death Pathways Triggered by Synthetic Cathinones

Caspase 3, 8, and 9 activity. The activity of caspases 3, 8, and 9 was determined in the cytoplasmatic fractions of primary cultured hepatocytes after a 24 h exposure to MDMA or the cathinone derivatives, as previously described (Valente *et al.*, forthcoming). Results were obtained from five independent experiments, run in duplicate. Protein contents on the cell lysate were measured using the Bio-Rad RC DC protein assay kit (Hercules, CA, USA), with albumin as the standard.

Hoechst 33342/propidium iodide fluorescent staining. Hepatocytes undergoing apoptosis or necrosis were identified based on nuclear morphological changes, using a cell-permeant nuclear counterstain that emits blue fluorescence when bound to DNA, Hoechst 33342, and a membrane impermeant nuclear dye that emits red fluorescence only in dead cells, PI (Valente *et al.*, forthcoming).

Statistical Analysis

Curves of normalized mortality values as a function of concentration, obtained through the MTT reduction assay, were constructed and analyzed as described by Dias da Silva *et al.* (2014), with a modified logit function applied as follows: $Y = \theta_{max}/(1 + \exp(-\theta_1 - \theta_2 \times \log(x)))$, where θ_{max} is the maximal observed effect, x is the concentration of the test drug, θ_1 is the parameter for the location, and θ_2 is the slope parameter. Statistical uncertainties are expressed as 95% confidence intervals (CI). Additionally, comparisons between curves (θ_{max}, θ_1 and θ_2) were performed using the extra sum-of-squares *F*-test. For further assays, results are presented as mean ± standard error of the mean (SEM). Normality of the data distribution was assessed by the D'Agostino and Pearson omnibus normality test. Multiple comparisons within each compound (concentration as a variable) or between each synthetic cathinone and MDMA were performed through one-way ANOVA analysis, followed by Fisher's LSD *posthoc* test. Non-linear curve fitting and all statistical calculations were performed using GraphPad Prism 6 (version 6.01) for Windows. *P*-values lower than .05 were considered statistically significant.

RESULTS

PRH Is a More Suitable In Vitro *Model than HepaRG to Study the Hepatotoxicity Elicited by Synthetic Cathinones*

The hepatotoxic potential of methylone, MDPV, pentedrone and 4-MEC was assessed in PRH and HepaRG cells through the MTT reduction assay. Figure 1 presents the concentration–response curves for each drug in the two used *in vitro* models. A summary of the calculated half maximal effective concentrations (EC50) of each compound, and other parameters from the nonlinear regression fit are provided in Supplementary Table S1. HepaRG cells proved to be significantly more resistant to the toxicity elicited by all five drugs, with curve fits substantially shifted to the right of PRH ones ($P < .0001$). For all four cathinones, but not MDMA, HepaRG cells presented steeper curves, with significantly higher slopes ($P < .01$ vs. PRH, when curves were compared for θ_2), which is representative of a higher variation of mortality within smaller concentration ranges in this *in vitro* model.

In order to determine whether the discrepant results between the two hepatic cellular models were due to differences in their intrinsic metabolic capacities, an additional study was conducted in the presence of quinidine, a CYP2D6 inhibitor.

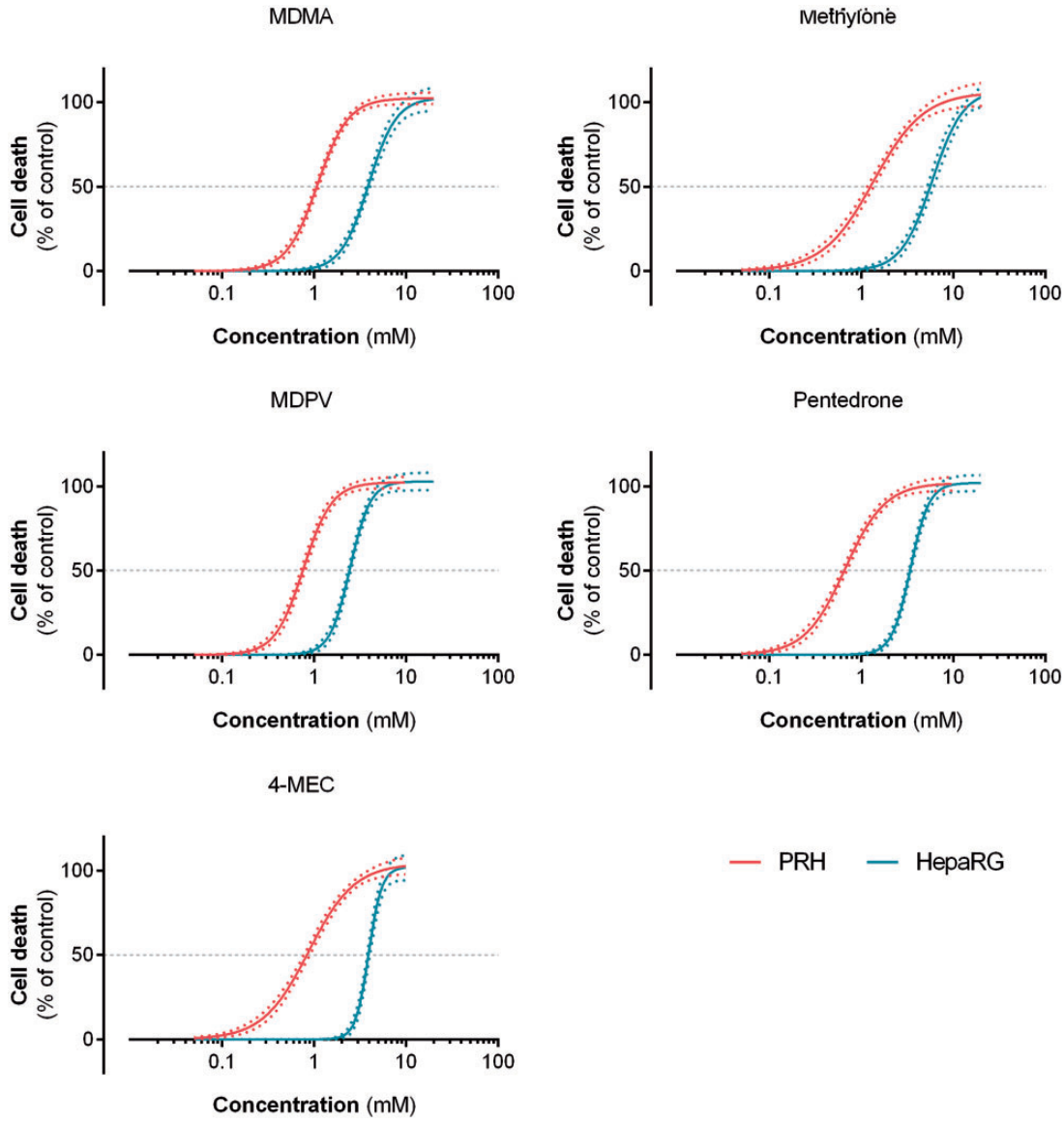


FIG. 1. Nonlinear regression models for the cell death induced by MDMA, methylone, MDPV, pentedrone, and 4-MEC in PRH and HepaRG cells, as evaluated by the MTT reduction assay after 24 h exposure. The mean effects were fitted to the logit function. Dotted lines represent the 95% confidence band of each fit. Results were obtained from at least four independent experiments, performed in triplicate.

As depicted in Table 2, quinidine partially, but significantly, reversed cell death induced by cathinone derivatives in PRH, with a significant increase in MTT reduction for 1.6 mM methylone, MDPV, and pentedrone ($P < .0001$). No significant differences were found between HepaRG cells exposed to the cathinones in the presence or absence of the CYP2D6 inhibitor (data not shown).

Due to the higher sensitivity of PRH to the cytotoxicity elicited by the tested cathinones, all further toxicological evaluations were carried out in this cell model.

Synthetic Cathinones Elicit Hepatotoxicity in Vitro in a concentration-Dependent Manner, with Variable Potency among Derivatives

Exposure of cells to synthetic cathinones resulted in concentration-dependent hepatotoxicity. However, cathinone derivatives displayed different toxic potencies, as follows: pentedrone ≈ MDPV > 4-MEC > methylone. Methylone was the least cytotoxic drug in both cell models, with EC50 values of 1.262 and 5.623 mM in PRH and HepaRG cells, respectively. MDPV and pentedrone were the most toxic compounds in HepaRG cells (EC50 of 2.432 and 3.405 mM) and PRH (EC50 of 0.756 and 0.656 mM, respectively). For both models, these two cathinones were even more hepatotoxic than MDMA (EC50 of 3.854 and 1.070 mM in HepaRG and PRH, respectively; $P < .05$). The toxic potential of 4-MEC was similar to MDMA in HepaRG cells (EC50 of 3.905 mM), but significantly higher in the primary culture (EC50 of 0.835 mM; $P < .01$).

In order to validate MTT reduction data obtained for PRH model, LDH leakage was assessed, as an unbiased test and indicator of cell membrane integrity, under equal experimental conditions. Results obtained from the LDH leakage assay are presented in Figure 2. In accordance to the MTT reduction data, a concentration-dependent increase in extracellular LDH was observed for all compounds. At 1.6 mM, after a 24 h exposure period, MDPV induced an increase on LDH leakage significantly

TABLE 2. Cell viability, as evaluated by the MTT reduction assay after a 24 h exposure of PHR to MDMA, methylone, MDPV, pentedrone and 4-MEC individually (1.6 mM), or in combination with 10 μM quinidine, 1 mM AA, 1mM NAC, or 100 μM Ac-LETD-CHO

Compound	Individual	+ 10 μM quinidine	+ 1 mM AA	+ 1 mM NAC	+ 100 μM Ac-LETD-CHO
MDMA	26.47 ± 1.96	30.79 ± 2.41	39.82 ± 1.51****	33.57 ± 3.06*	34.29 ± 1.26*
Methylone	38.70 ± 1.58	55.44 ± 1.81****	45.33 ± 1.87*	45.76 ± 1.88**	45.70 ± 2.44**
MDPV	4.92 ± 0.45	10.36 ± 0.86****	12.47 ± 1.17****	5.51 ± 0.92	11.85 ± 0.69****
Pentedrone	8.43 ± 0.62	19.46 ± 2.10****	17.77 ± 1.37****	13.44 ± 1.40**	18.86 ± 1.75****
4-MEC	17.72 ± 1.04	20.84 ± 2.46	23.48 ± 1.93*	18.25 ± 1.82	26.92 ± 3.07***

Results are presented as mean ± SEM. *P < 0.05; **P < .01; ***P < .001; ****P < .0001 versus individual compounds.

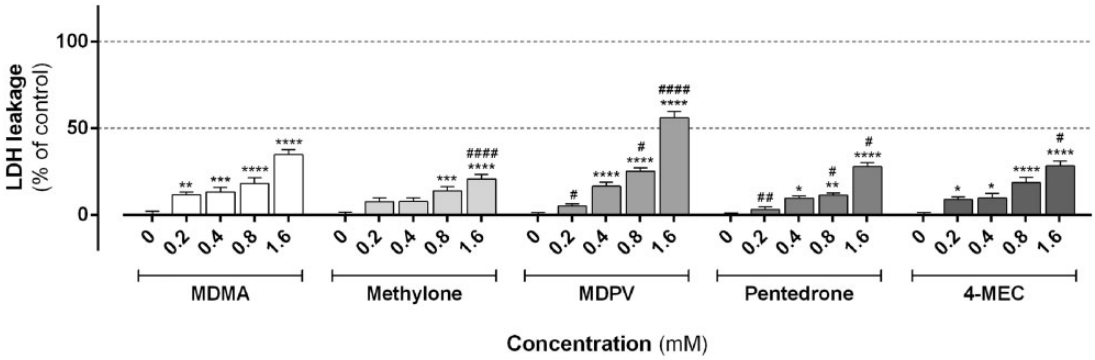


FIG. 2. Extracellular LDH quantification, 24 h after exposure of PHR to MDMA, methylone, MDPV, pentedrone, and 4-MEC. Results were obtained from 6 independent experiments, performed in triplicate. *P < .05, ** P < .01, *** P < .001, **** P < .0001 versus control. # P < .05, ## P < .01, #### P <.0001 versus MDMA.

higher than MDMA (56.01 ± 3.61% for MDPV vs. 34.78 ± 2.82% for MDMA, P < .0001). At this concentration, 4-MEC, pentedrone and methylone were, in this order, significantly less effective than MDMA (28.35 ± 2.92, 27.86 ± 2.28, and 20.68 ± 2.70%, respectively).

Synthetic Cathinones Have a Strong Reducing Potential

The reducing power assay is often used to evaluate the overall capability of a compound to donate an electron, as compounds with higher reducing power have superior electron-donor ability (Kohen and Nyska, 2002). As depicted in Figure 3, all four cathinones presented a concentration-dependent reducing potential, in the following order of potency: pentedrone ≈4-MEC > methylone > MDPV. To attain a reducing power of 0.5 (Abs = 0.500), the concentrations of cathinones required were 4.161 ± 0.009, 4.230 ± 0.010, 6.047 ± 0.026, and 40.451 ± 0.700 mM for pentedrone, 4-MEC, methylone and MDPV, respectively. MDMA showed an almost negligible reducing power, with an absorbance of 0.042 ± 0.001 for the highest concentration tested, 50 mM.

Synthetic Cathinones Trigger Oxidative Stress in PRH, and Cell Death Is Partially Reversed by Antioxidants

In order to determine the role of oxidative stress in cathinones-induced hepatotoxicity, the formation of ROS and RNS and the intracellular levels of GSH and GSSG were measured in PRH exposed to the cathinone derivatives for 24 h. Our data show that all four cathinone derivatives were capable of stimulating the production of ROS and RNS (Fig. 4). MDMA and MDPV were the least effective compounds, eliciting identical increase of ROS and RNS, only significant at the higher concentration tested (1.23 ± 0.04 and 1.24 ± 0.07 fold increase over control, P < .0001). Methylone, 4-MEC and pentedrone induced a significant increase of ROS and RNS production, already observable at a

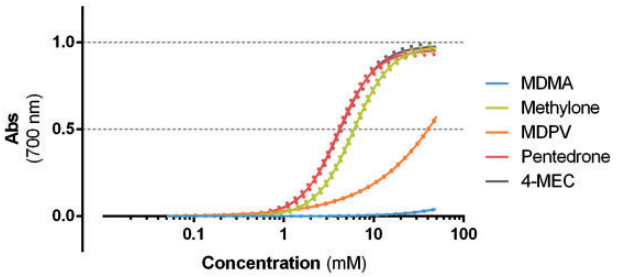


FIG. 3. Nonlinear regression models for the reducing power of MDMA, methylone, MDPV, pentedrone, and 4-MEC. The mean effects were fitted to the logit function. Dotted lines represent the 95% confidence band of each fit. Results were obtained from three independent experiments, performed in duplicate.

concentration as low as 0.4 mM (1.10 ± 0.02, 1.14 ± 0.03, and 1.17 ± 0.02-fold increase over control, respectively, P < .05). At 1.6 mM, ROS and RNS production for pentedrone was extensively greater when compared with control (1.56 ± 0.07 fold increase over control, P < .0001) and to MDMA at the same concentration. As depicted in Figure 5A, and in accordance to the prior results, pentedrone was also the most effective compound in decreasing the intracellular levels of GSH (23.74 ± 2.19 at 1.6 mM vs. 43.89 ± 4.24 nmol/mg of protein in control cells, P < .0001), which is a primary cellular antioxidant. However, the GSH depletion induced by pentedrone was not accompanied by corresponding increases in GSSG levels (Fig. 5B). For the other 4 drugs, a general decrease in GSH levels was followed by an increase in the oxidized form, in an apparent concentration-dependent manner. Nonetheless, no significant differences were observed in the GSH intracellular concentration between any of the synthetic cathinones and MDMA.

To further define the role of oxidative stress in cathinones-induced cell death, two well-described antioxidants were tested in combination with the drugs at 1.6 mM, the concentration at

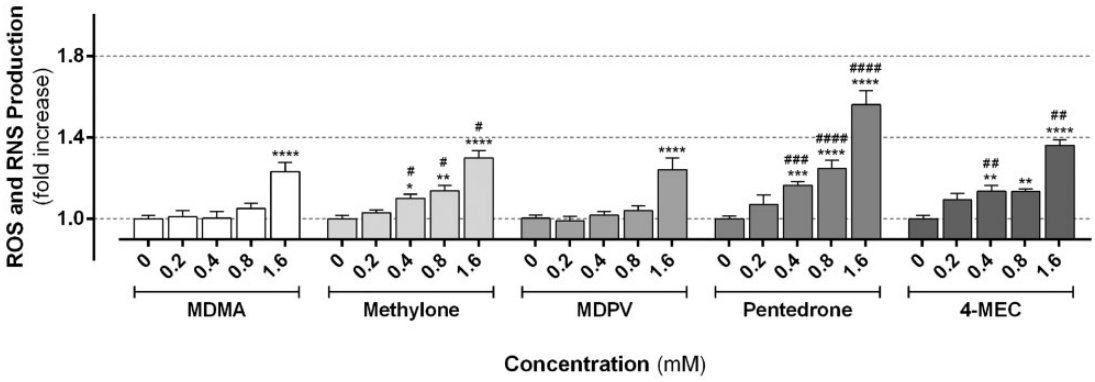


FIG. 4. ROS and RNS production in PHR exposed to MDMA, methylone, MDPV, pentedrone, and 4-MEC, for 24 h. Results were obtained from five independent experiments, performed in triplicate. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$ versus control. # $P < .05$, ## $P < .01$, ### $P < .001$, #### $P < .0001$ versus MDMA.

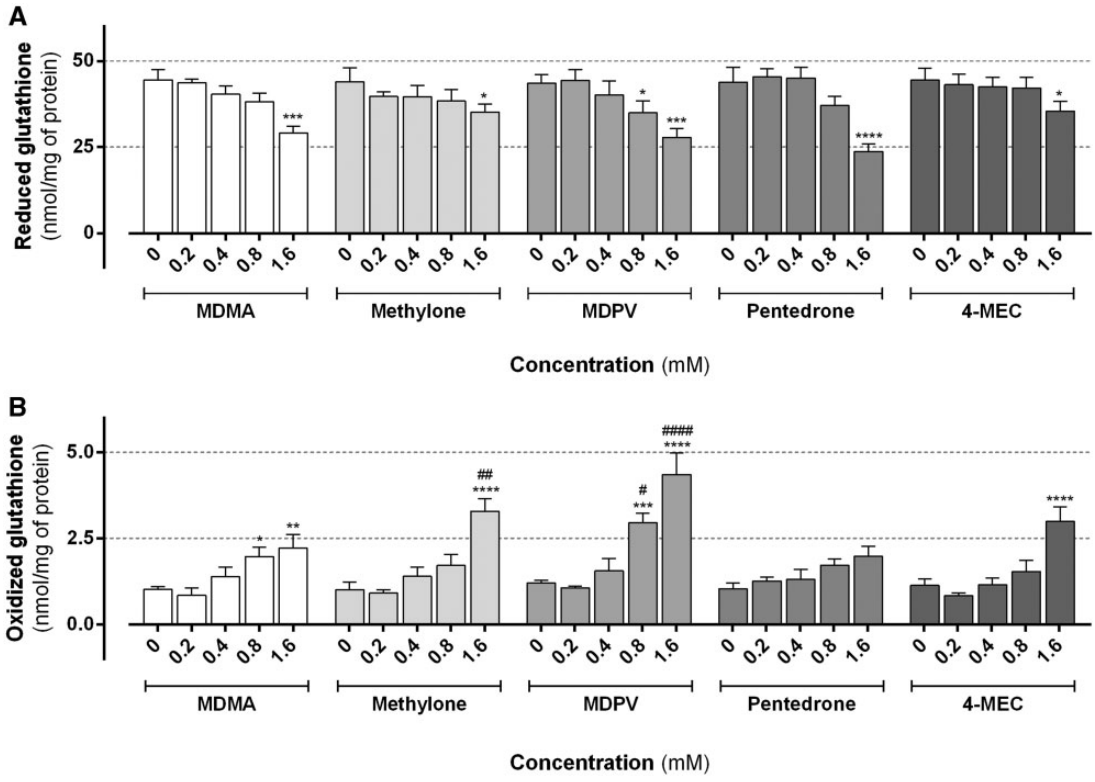


FIG. 5. Intracellular levels of **(A)** GSH and **(B)** GSSG in PHR exposed MDMA, methylone, MDPV, pentedrone and 4-MEC, for 24 h. Results were obtained from five independent experiments, performed in duplicate. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$ versus control. # $P < .05$, ## $P < .01$, #### $P < .0001$ versus MDMA.

which all drugs induce significant increase in ROS and RNS generation and depletion of GSH levels. Results are presented in Table 2. Individually, 1 mM AA and NAC did not show toxicity towards PRH (data not shown). Incubation with AA significantly reversed the cytotoxicity induced by all four synthetic cathinones and MDMA ($P < .05$). This antioxidant effect was more effective with MDMA, pentedrone and MDPV, with a gain of about 13, 9, and 8% in cell viability ($P < .0001$ vs. individual compounds at 1.6 mM), respectively. On the other hand, NAC partially attenuated MDMA, methylone and pentedrone-induced cell death, with a decrease in cell death of about 7% for MDMA and methylone, and 5% for pentedrone ($P < .05$ vs. individual compounds at 1.6 mM). Despite a slight increase in cell viability in PRH exposed to MDPV and 4-MEC in the presence of NAC, this protective effect did not reach statistical significance.

Synthetic Cathinones Disrupt the Hepatocellular Energetic Status

Mitochondria participate in several vital cellular functions, including ATP generation as a source of energy for cells. As depicted in Figure 6, after 24 h of exposure, MDMA, MDPV, and pentedrone were able to significantly reduce ATP intracellular levels at the highest concentration tested, 1.6 mM ($P < .05$). No significant effects were observed with methylone or 4-MEC at any concentrations. MDPV was the most effective drug, inducing a decrease of almost 60% on ATP levels, from 20.65 ± 1.95 in control cells to 8.55 ± 1.67 nmol/mg of protein at 1.6 mM ($P < .001$). At the same concentration, pentedrone and MDMA induced a decline of about 40% of ATP levels. Nonetheless, no significant differences were observed among 1.6 mM

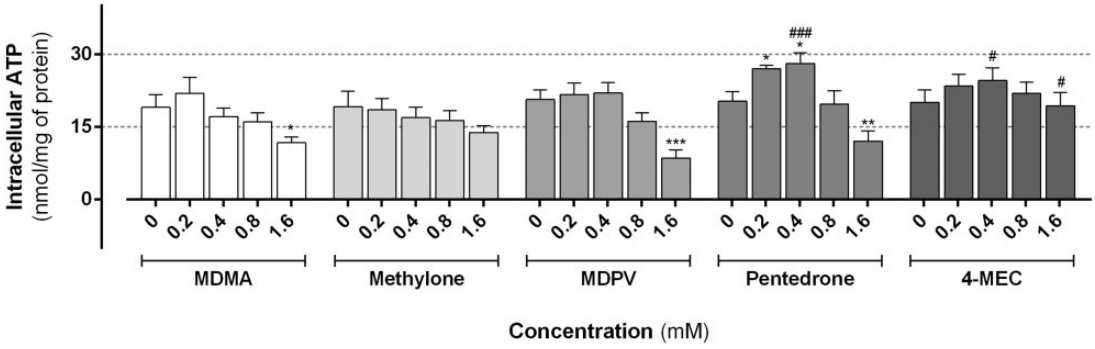


FIG. 6. Intracellular levels of ATP in PHR exposed to MDMA, methylone, MDPV, pentedrone, and 4-MEC, for 24 h. Results were obtained from 5 independent experiments, performed in duplicate. * $P < .05$, ** $P < .01$, *** $P < .001$ versus control. # $P < .05$, #### $P < .0001$ versus MDMA.

pentedrone, MDPV and MDMA. Of note, at lower concentrations (0.2 and 0.4 mM) an increase in ATP production was observed for pentedrone ($P < .05$ vs. control).

Synthetic Cathinones Induce Apoptotic Cell Death, Which Is Partially Reversed by a Caspase Inhibitor

To determine whether synthetic cathinones-induced hepatotoxicity involves programed cell death, the activation of caspase 3 was evaluated as a main event leading to apoptosis. In order to establish the involvement of extrinsic and/or intrinsic pathways, caspases 8 and 9 activities were further analyzed. All four cathinone derivatives induced the activation of the effector caspase, caspase 3, in a concentration-dependent manner (Fig. 7A). For methylone and 4-MEC, this activation was only significant at the higher concentration tested, with an increase of about 60% over control cells ($P < .0001$), but no significant differences were found when compared with MDMA. Although MDMA significantly activated caspase 3 already at 0.4 mM (130.89 ± 11.54%, $P < .05$ vs. control cells), pentedrone and MDPV were the most effective compounds at the higher concentration, with an activation of 254.03 ± 23.45 and 213.60 ± 13.85%, respectively, at 1.6 mM ($P < .0001$ vs. control cells), which was significantly greater than that observed with MDMA at the same concentration (177.51 ± 20.06%, $P < .05$). Similar tendency was observed for caspases 8 and 9 (Figs. 7B and C), with a more pronounced activation with pentedrone and MDPV. At 1.6 mM, these two derivatives led to an activation of 207.80 ± 18.22 and 165.37 ± 5.60% of caspase 8, and 249.14 ± 15.83 and 190.42 ± 7.04% of caspase 9 ($P < .0001$ vs. control cells, $P < .05$ vs. 1.6 mM MDMA). Results are consistent with the activation of both intrinsic (caspase 9) and extrinsic (caspase 8) apoptotic pathways. Thus, the protective potential of Ac-LETD-CHO, a caspase inhibitor known to exert inhibitory effects on caspases 8 and 9, was evaluated. When tested individually, 100 μM Ac-LETD-CHO presented no cytotoxicity (data not shown). The combination of the caspase inhibitor with each compound induced a modest but significant increase of cell viability of about 7–10% when compared with the individual drugs at 1.6 mM ($P < .05$).

The triggering of apoptosis was also demonstrated with Hoechst 33342/PI staining, with evident changes in the chromatin morphology of cells exposed to the synthetic cathinones (Supplementary Figure S1). In control cells, nuclei appear fairly round, with regular contours, and large in size. With the increase in concentration of the drugs, it is evident a reduction of the nuclei size, distinctive of chromatin condensation (eg, 1.6 mM pentedrone compared with control cells), and the presence of pyknotic nuclei, characteristic of early (without PI label, green arrows) and late (with PI label, orange arrows) apoptotic events. In accordance with our data on caspase 3 activation, apoptotic events in MDMA-exposed cells were apparent already at 0.4 mM (Supplementary Figure S1). Nonetheless, a general concentration-dependent increase of apoptotic cells was observed for all compounds, though less evident with methylone. Necrotic cells (large PI-labeled nuclei, red arrows) were more evident at the two highest concentrations tested (0.8 and 1.6 mM).

DISCUSSION

Little is currently known about the potential harms arising from the use of this new generation of cathinone-derived designer drugs. Due to chemical similarity to long-studied amphetamines (particularly MDMA) and the use as alternative for these drugs, similar pharmacodynamics and toxicological mechanisms for β-keto amphetamine-derived designer drugs could be postulated. Thus, there is a great need to study these cathinone derivatives at a fundamental level by comparing their potencies with related amphetamines in order to make risk assessments. In our study, all four synthetic cathinones showed significantly different toxicological potencies in both PRH and HepaRG cells. Methylone was the least cytotoxic substance in both hepatic *in vitro* models, significantly less potent than its non-keto analogue, MDMA, whereas MDPV and pentedrone were more potent than MDMA. 4-MEC was also more toxic than MDMA in PRH, but no significant differences were observed between these two compounds in HepaRG. These results may be partially explained by chemical properties and structures of the compounds in study. Methylone (βk-MDMA) presents a lower log *P* value (1.08) when compared with its related amphetamine MDMA (1.98), which indicates lower lipophilicity as expected from the presence of the β-keto group. On the other hand, the presence of the pyrrolidine ring in cathinones like MDPV greatly reduces their polarity (Coppola and Mondola, 2012), resulting in a greater diffusion of this derivative through cell membranes. In fact, MDPV has the highest log *P* value (2.63) of all cathinones in study, being even higher than MDMA, which is in conformity with the significantly higher increase in toxicity, as evidenced by the LDH leakage assay (Figure 2). Data on extracellular LDH also supports the concentration-dependent pattern observed with the MTT reduction assay. However, the magnitude of loss of integrity of the plasma membrane, as determined by the LDH leakage assay, was lower than the decrease of mitochondrial function, given by the MTT reduction assay. This result was somehow expected since mitochondrial dysfunction often precedes membrane damage, thus explaining the greater sensitivity of the MTT assay.

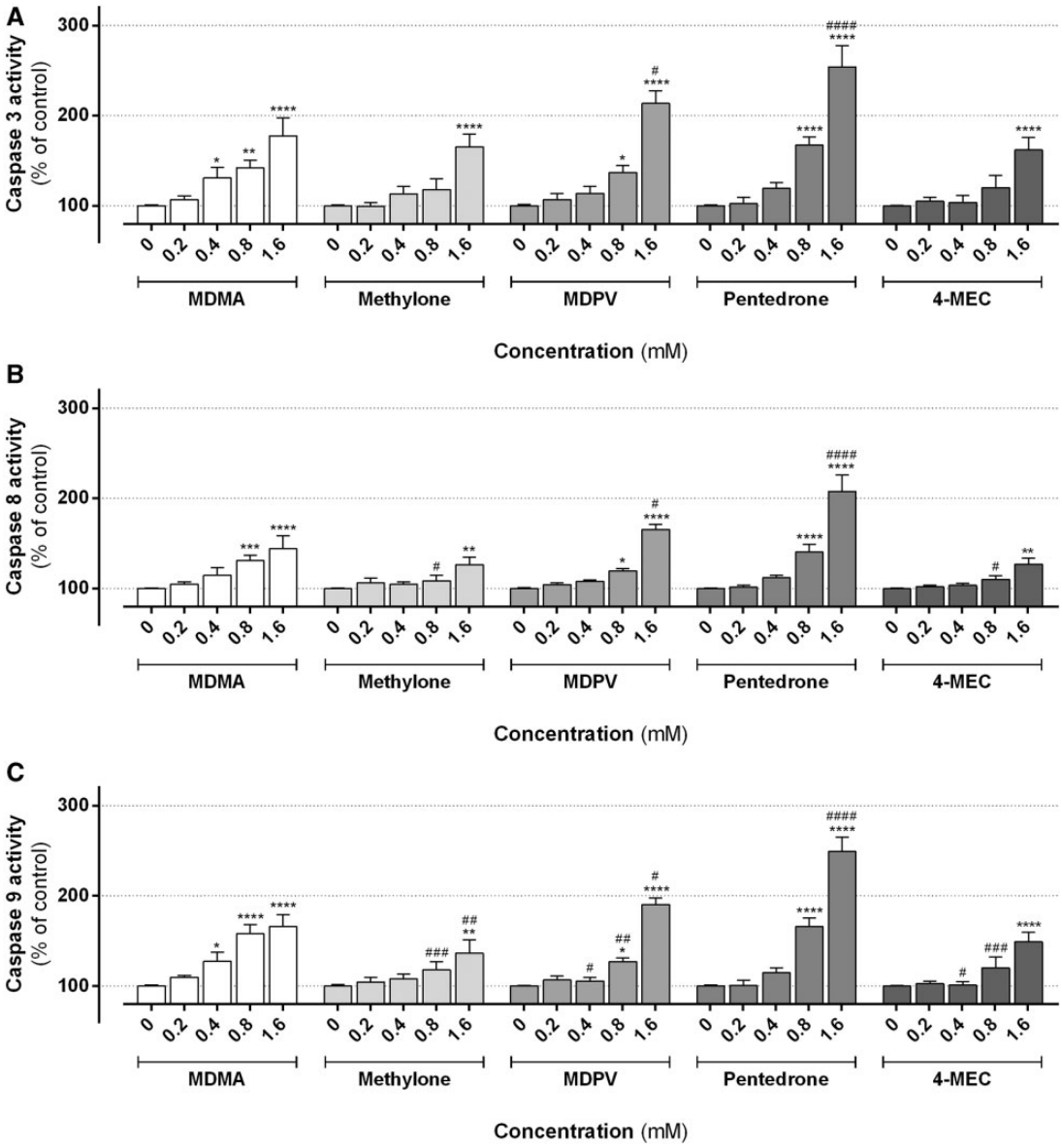


FIG 7. Percentage of increase in caspases (A) 3, (B) 8, and (C) 9 activity in PHR exposed to MDMA, methylone, MDPV, pentedrone and 4-MEC, for 24 h. Results were obtained from four independent experiments, performed in duplicate. * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$ versus control. # $P < .05$, ## $P < .01$, ### $P < .001$, #### $P < .0001$ versus MDMA.

The hypothesis of a chemical contribution to the observed cytotoxic effects was further evaluated through the measurement of the redox potential of the cathinone derivatives. As depicted in Figure 3, all four cathinones presented concentration-dependent reducing power. Conversely, MDMA displayed virtually no reducing ability. Accordingly, with a pK_a value of 10.14 (Table 1), MDMA is the stronger base, which means that under physiologic pH (7.4) it is mostly found in the protonated form, and thus detains low electron-donor capacity. When comparing MDMA and its β-keto analogue, the combination of lower pK_a (7.96) and the ability to undergo keto-enol tautomerization (Coppola and Mondola, 2012) may contribute to the higher reducing power of methylone. This is also valid for 4-MEC and pentedrone. On the other hand, despite being the weaker base under study (pK_a value of 7.31), MDPV presents a relatively low reducing potential, which may be a consequence of molecular stabilization by the presence of the pyrrolidine group (Leffler *et al.*, 2014). Our results are in accordance with data from the work of den Hollander *et al.* (2014), which showed significant differences on redox reactivity among several β-keto and non-keto amphetamines, using a tetrazolium redox indicator.

Results show substantial differences between the two cellular models regarding the susceptibility to drug-induced cytotoxicity. HepaRG cells were significantly less sensitive to the toxicity elicited by cathinone derivatives, with EC50 values about 3 to 5 times higher than those observed in PRH (Supplementary Table S1). Under specific culture conditions, the human hepatoma HepaRG cells represent a highly differentiated and metabolically competent liver cell model, capable of expressing both phase I and II drug-metabolizing enzymes as observed *in vivo* (Guillouzo *et al.*, 2007). For this reason, this model has been used as a suitable alternative to human hepatocytes for the study of well-known hepatotoxic compounds (Antherieu *et al.*, 2011; McGill *et al.*, 2011; Sharanek *et al.*, 2014).

However, despite the general advantages of using an immortalized cell line, such as the ease of manipulation, the ability to be cryopreserved and the reduced data variability, the expression of cytochrome P450 enzymes in HepaRG cells is, for most isoenzymes, significantly lower than that observed in freshly isolated human hepatocytes (Aninat *et al.*, 2006), which greatly affects the translation of results into the *in vivo* situation. This is particularly critical for compounds with CYP2D6-dependent metabolism, since this hepatoma cell line was derived from a CYP2D6 poor metabolizer patient (Guillouzo, *et al.*, 2007). In fact, recent evidence suggests that CYP2D6 is the main responsible enzyme for the *in vitro* phase I metabolism of synthetic cathinones (Negreira *et al.*, 2015; Pedersen *et al.*, 2013a, b), which may explain the higher resistance of HepaRG to the synthetic cathinones-induced cytotoxicity. This lower sensitivity of the hepatoma cell line was also observed with MDMA, for which CYP2D6-mediated bioactivation into hepatotoxic metabolites is fully accepted (Carmo *et al.*, 2006; Carvalho *et al.*, 2012). To ascertain whether the CYP2D6 metabolism could explain the higher susceptibility of PRH, both cell models were co-incubated with the cathinones and the CYP2D6 inhibitor, quinidine. A significant reversion in cell death was observed in PRH for methylone, MDPV and pentedrone, while, as expected, no effect was seen in HepaRG cells. Taken together, these findings indicate that PRH is a more suitable *in vitro* model for toxicological studies on synthetic cathinones.

The synthetic cathinones under study triggered oxidative stress as evidenced by enhanced generation of ROS and RNS (Figure 4), and decreases in intracellular GSH levels (Figure 5A). Methylone, 4-MEC and pentedrone were the most potent drugs regarding the formation of ROS and RNS, whereas MDPV and MDMA were only effective at the highest concentration tested. Interestingly, our results show that cathinones-induced ROS and RNS formation appears to be correlated with their reducing potential (electron donor capacity), as derivatives with higher reducing power (methylone, pentedrone, and 4-MEC) elicit greater ROS and RNS production. It is possible that increased reducing power may lead to greater redox cycling of the cathinone metabolites, thereby promoting greater ROS generation, lipid peroxidation, and protein adduction. However, further studies are required to ascertain the contribution of redox properties of the studied drugs to the respective toxicological effects. It is also acknowledged that oxidative stress arises from the disruption of the mitochondrial respiratory chain, contributing to an increase in intracellular ROS and RNS formation (Brookes *et al.*, 2004). Importantly, recent studies have revealed that mephedrone and methylone induce mitochondrial respiratory dysfunction in neuronal cells (den Hollander, *et al.*, 2014, 2015), which is also a recognized mechanism underlying amphetamines-induced toxicity (Carvalho *et al.*, 2012). Furthermore, MDMA is known to undergo extensive hepatic cytochrome P450-mediated bioactivation, with formation of catechols and further oxidation to *o*-quinones, which are highly reactive molecules that may enter redox cycling and lead to the production of ROS and RNS (Carvalho *et al.*, 2010). Since the formation of catechols through *o*-demethylenation was shown to be one of the main metabolic pathways for methylone and MDPV (Pedersen *et al.*, 2013a; Strano-Rossi *et al.*, 2010), it could be anticipated that a similar bioactivation pathway takes place with cathinone derivatives containing the methylenodioxy ring. Nonetheless, further work is needed to elucidate the exact contribution of these mechanisms to the augmented formation of ROS/RNS induced by cathinones.

GSH is a major endogenous antioxidant defense, being responsible for numerous functions while protecting the liver against drug-induced damage (Chen *et al.*, 2013). Since one of these functions is the direct scavenging of superoxide anion and hydroxyl radical, GSH depletion is a foreseeable outcome from the increase in ROS and RNS production. Accordingly, all four cathinones induced a significant decrease in GSH intracellular levels at the higher concentration tested (Figure 5A). Pentedrone was the most effective compound, but no significant increase in GSSG levels was observed after 24 h. The *in vitro* formation of glutathionyl adducts with the demethylenated metabolites of methylenedioxy β-keto amphetamines, including MDPV and methylone, was recently demonstrated (Meyer *et al.*, 2014). However, there is no evidence supporting the ability of cathinones lacking the methylenedioxy ring, such as pentedrone and 4-MEC, to undergo metabolic pathways that result in the formation of conjugates with GSH. For this reason, the absence of a significant increase in GSSG intracellular levels for pentedrone may be due to an ATP-dependent export of the formed GSSG into the extracellular space (Leier *et al.*, 1996). The formation of ROS and RNS and the GSH depletion are well-acknowledged toxicological pathways of MDMA-induced hepatotoxicity (Carvalho *et al.*, 2010), but little is known on the ability of synthetic cathinones to induce oxidative stress. However, some recent studies demonstrated that the cathinone derivative 4-methylmethcathinone (mephedrone) elicits oxidative stress in the brain of rats and mice, characterized by increased lipid peroxidation and altered enzymatic antioxidant systems (Budzynska *et al.*, 2015; Ciudad-Roberts *et al.*, 2016; Lopez-Arnau *et al.*, 2015).

Since oxidative stress appears to underlie the observed hepatotoxicity, it is expected that antioxidants prevent or partially revert the cell death induced by the cathinones. In fact, in our study, 1 mM AA significantly reversed cell death induced by MDMA and all four cathinones, but more pronouncedly for MDMA and its β-keto analogue, while protection with 1 mM NAC was significant only for MDMA, methylone and pentedrone (Table 2). Accordingly, our group has previously demonstrated the protective effects of AA and NAC against the hepatotoxicity elicited by the main MDMA catecholic metabolite, *N*-methyl-α-methyldopamine (Carvalho *et al.*, 2004), as well as the neuroprotection of NAC against the cytotoxicity induced by this metabolite and the GSH conjugate, 5-GSH-α-methyldopamine (Capela *et al.*, 2006). The protective effect of AA is widely acknowledged, and relies on its ability to scavenge ROS and RNS and to regenerate other antioxidants, such as α-tocopherol (Maxwell, 1995). NAC exerts antioxidant effects by acting either directly, as a ROS and RNS scavenger, or indirectly, as a source of cysteine, the precursor for the rate-limiting step of the synthesis of glutathione (Firuzi *et al.*, 2011). This latter function is important when the liver is depleted of GSH, becoming more vulnerable to oxidative damage, which is the case of the synthetic cathinones in this study. Although MDPV is one of the most hepatotoxic compounds, triggering significant depletion of GSH intracellular levels at 1.6 mM, NAC failed to exhibit cytoprotection in the presence of this substance at this concentration. A plausible explanation for these results would be the formation of cytotoxic thioether conjugates of MDPV with NAC and GSH, as it is known to occur with MDMA (Jones *et al.*, 2005), but further studies with MDPV conjugates are required to verify this hypothesis. Since 1.6 mM 4-MEC did not lead to a depletion of GSH or an increase in ROS and RNS production as great as pentedrone, the induction of these oxidative stress pathways may be less preponderant in 4-MEC-induced hepatotoxicity at the conditions tested, thereby explaining the lack of protective effect by NAC.

Used by cells to execute energy-requiring processes, ATP is the primary energy currency, and since mitochondria are the main source of ATP, mitochondrial ATP generation is essential for the maintenance of cellular integrity (Brookes *et al.*, 2004). Our results show that MDPV and pentedrone, as well as MDMA, elicited a significant decline in ATP synthesis after 24 h (Figure 6). This decrease occurred at a concentration where the GSH depletion and the formation of ROS and RNS were significantly increased, especially for pentedrone, suggesting that oxidative stress may be involved in mitochondrial dysfunction. However, despite the decrease in GSH levels observed for methylone and 4-MEC, the mitochondrial damage appears to be insufficient to hamper ATP synthesis with these two derivatives, at least at the range of concentrations tested. The involvement of mitochondria in amphetamines-induced toxicity has been thoroughly reviewed (Barbosa *et al.*, 2015; Carvalho *et al.*, 2012). In response to oxidative injury, the mitochondria undergo extensive changes, including the permeabilization of the mitochondrial membrane, with release of pro-apoptotic proteins into the cytosol, such as cytochrome *c* (Brookes *et al.*, 2004). Once in the cytosol, cytochrome *c* initiates the complex signaling cascade that characterizes the intrinsic mechanisms of apoptosis, with activation of caspase 9 and downstream executioner caspases 3 and 7 that, in turn, cleave intracellular substrates, causing the morphological and biochemical changes that ultimately end in cell death (Tait and Green, 2010; Tsujimoto, 2003). Our data show that all cathinones prompt apoptosis, with activation of both caspases 3 and 9 (Figs. 7A and C). The activation of extrinsic apoptosis was also evaluated through the measurement of caspase 8 activation. This extrinsic pathway is mitochondrion-independent, and it is initiated by the binding of xenobiotics to death receptors located in the surface of cell membrane, with subsequent activation of caspase 8, and further activation of the executioner caspases, or indirect activation of the intrinsic apoptotic pathway (McIlwain *et al.*, 2013). As seen in Figure 7B, all five compounds elicited the activation of caspase 8 in an identical pattern to that observed for caspases 3 and 9. Methylone and 4-MEC were the least effective compounds, which may be expected from the putative inferior potency to induce mitochondrial dysfunction. MDPV and pentedrone, the most hepatotoxic drugs in study, and the most potent compounds concerning ATP depletion and oxidative stress stimulation, respectively, were the most effective triggers of apoptosis, as well. Hoechst 33342/PI staining further supports these findings, with an apparent increase in apoptotic events (green and orange arrows) in a concentration dependent manner (Supplementary Figure S1). This increase was less evident for methylone, which is also the least hepatotoxic drug in study. The presence of necrotic events (red arrows) was also noted at higher concentrations. It is important to note that necrosis is a degradative process that may occur as a primary event of cell death or secondary to apoptosis. Based on our findings showing the absence of necrotic events at lower concentrations of the studied drugs, it seems that the observed LDH release in cells exposed to low concentrations is more likely due to secondary necrosis from late apoptosis. Additionally, the decrease in MTT reduction, a marker of mitochondrial dysfunction, under the same experimental conditions, further supports apoptosis as a main pathway of cell death. For this reason, it was expected that caspase inhibition would detain protective properties against cathinones-induced toxicity. In fact, 100 μM Ac-LETD-CHO, a caspase 8 and 9 inhibitor, significantly reduced the cell death elicited by all cathinones (Table 2). MDPV was previously shown to prompt apoptosis in neonatal mouse brain after acute administration (Adam *et al.*, 2014), whereas the overexpression of the anti-apoptotic Bcl-2 protein partially prevented the cytotoxicity elicited by mephedrone in neuronal cells (den Hollander *et al.*, 2015).

One major limitation of this study is the use of relatively high concentrations of synthetic cathinones when compared with the micromolar levels found in blood samples from 'bath salts'-related intoxications (Gil *et al.*, 2013; Kesha *et al.*, 2013; Pearson *et al.*, 2012; Sykutera *et al.*, 2015). Nonetheless, due to the toxicokinetics, and especially drug distribution and tissue accumulation, it should be noted that the drug concentration to which the liver is actually exposed may be much higher than that found in the blood, to similarly to what was determined for MDMA (García-Repetto *et al.*, 2003). In fact, it was recently shown that pentedrone and MDPV reach liver-to-blood ratios up to 11 and 23, respectively (Marinetti and Antonides, 2013; Sykutera *et al.*, 2015). Furthermore, it is important to stress that the present work involves a comparative mechanistic study, and the obtained data should be regarded as a means of improving the understanding of the cellular mechanisms that may be involved in the *in vivo* effects of synthetic cathinones.

In conclusion, the present work provided evidence of the hepatotoxicity of cathinone derivatives in *in vitro* models, whose mechanisms may help to understand liver damage reported in humans. Variations in magnitude among the different analogues may be attributable to their chemical properties, including lipophilicity and reducing power, as more lipophilic compounds present greater hepatotoxic potential, and more powerful reducing cathinones elicit enhanced formation of reactive species. Furthermore, this study demonstrated for the first time a common mechanism for cathinones-induced hepatic injury involving oxidative stress and mitochondrial dysfunction. Finally, apoptosis appears to be the main pathway of cell death elicited by these novel drugs, with activation of both intrinsic and extrinsic mechanisms.

SUPPLEMENTARY DATA

Supplementary data are http://toxsci.oxfordjournals.org/

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ORGAN TOXICITY AND MECHANSIMS

3,4-Methylenedioxypyrovalerone (MDPV): in vitro mechanisms of hepatotoxicity under normothermic and hyperthermic conditions

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Abstract Synthetic cathinones have emerged in recreational drug markets as legal alternatives for classical amphetamines. Though currently banned in several countries, 3,4-methylenedioxypyrovalerone (MDPV) is one of the most commonly abused cathinone derivatives worldwide. We have recently reported the potential of MDPV to induce hepatocellular damage, but the underlying mechanisms responsible for such toxicity remain to be elucidated. Similar to amphetamines, a prominent toxic effect of acute intoxications by MDPV is hyperthermia. Therefore, the present in vitro study aimed to provide insights into cellular mechanisms involved in MDPV-induced hepatotoxicity and also evaluate the contribution of hyperthermia to the observed toxic effects. Primary cultures of rat hepatocytes were exposed to 0.2–1.6 mM MDPV for 48 h, at 37 or 40.5 °C, simulating the rise in body temperature that follows MDPV intake. Cell viability was measured through the MTT reduction and LDH leakage assays. Oxidative stress endpoints and cell death pathways were evaluated, namely the production of reactive oxygen and nitrogen species (ROS and RNS), intracellular levels of reduced (GSH) and oxidized (GSSG) glutathione, adenosine triphosphate (ATP) and free calcium (Ca^{2+}), as well as the activities of caspases 3, 8 and 9, and nuclear morphological changes with Hoechst 33342/PI double staining. At 37 °C, MDPV induced a concentration-dependent loss of cell viability that was accompanied by GSH depletion, as one of the first signs of toxicity, observed already at low concentrations of MDPV, with negligible changes on GSSG levels, followed by accumulation of ROS and RNS, depletion of ATP contents and increases in intracellular Ca^{2+} concentrations. Additionally, activation of caspases 3, 8, and 9 and apoptotic nuclear morphological changes were found in primary rat hepatocytes exposed to MDPV, indicating that this cathinone derivative activates both intrinsic and extrinsic apoptotic death pathways. The cytotoxic potential of MDPV and all the studied endpoints were markedly aggravated under hyperthermic conditions (40.5 °C). In conclusion, these data suggest that MDPV toxicity in primary rat hepatocytes is mediated by oxidative stress, subsequent to GSH depletion and increased ROS and RNS accumulation, mitochondrial dysfunction, and impairment of Ca^{2+} homeostasis. Furthermore, the rise in body temperature subsequent to MDPV abuse greatly exacerbates its hepatotoxic potential.

Keywords MDPV · Synthetic cathinones · Hepatotoxicity · Hyperthermia · Oxidative stress

Abbreviations

MDPV	3,4-Methylenedioxypyrovalerone
MDMA	3,4-Methylenedioxymethamphetamine
GSH	Reduced glutathione
GSSG	Oxidized glutathione
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
ATP	Adenosine triphosphate

Introduction

Synthetic cathinones are a broad group of novel psychostimulant substances, commonly known as 'bath salts',

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that originally appeared in the recreational drug markets as legal alternatives to classical illicit drugs, namely amphetamines and cocaine (for a comprehensive review on synthetic cathinones, please see Valente et al. 2014) Although recently regulated in several countries, cathinone derivatives are expanding into the illicit markets and, therefore, remain widely accessible in Internet Web sites and can also be purchased through street dealers (German et al. 2014; Johnson and Johnson 2014). This group of substances comprises structural derivatives of cathinone, a phenylalkylamine alkaloid naturally present in the khat plant (*Catha edulis*) (Valente et al. 2014). Cathinone is structurally related to amphetamine, differing only by the presence of a ketone group in the β-position of the alkylamine side chain (Fig. 1). For this reason, cathinone derivatives are often termed β-keto amphetamines. Based on the close chemical similarities, cathinone and cathinone-derived designer drugs share many of the pharmacological and behavioral characteristics commonly associated with the amphetamine psychostimulants, including increased locomotor activity (Aarde et al. 2013; Gatch et al. 2013; Jones et al. 2014; Lopez-Arnau et al. 2012), thermoregulatory disruption (Lopez-Arnau et al. 2015; Miller et al. 2013; Shortall et al. 2013) and the ability to produce discriminative stimulus effects (Fantegrossi et al. 2013; Gatch et al. 2013; Varner et al. 2013). From over seventy synthetic cathinone derivatives identified so far, 3,4-methylenedioxypyrovalerone (MDPV) has received notoriety as a major component of 'bath salts' products (EMCDDA-Europol 2014; EMCDDA 2015; Zuba and Byrska 2013), and is one of the most commonly abused derivatives worldwide by virtue of its amphetamine- and cocaine-like effects (Johnson and Johnson 2014; Murray et al. 2012). Structurally, MDPV contains both a 3,4-methylenedioxy ring attached to the phenyl group similar to the well-known 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy'), and a pyrrolidine ring in the side chain making it close to the psychostimulant pyrovalerone (Fig. 1). Not surprisingly, MDPV induces MDMA-like subjective effects, including increased energy, mild empathogenic effects and sociability, and increased mental stimulation and sexual drive (Coppola and Mondola 2012a; Deluca et al. 2009; Kesha et al. 2013).

Information on the toxicological properties of these emerging drugs of abuse is limited, but several cases of 'bath salts'-related intoxication and deaths have been reported over the last years (James et al. 2011; Murray et al. 2012; Wood et al. 2010). Most symptoms are consistent with sympathetic stimulation, including tachycardia, vasoconstriction, hypertension, hyperthermia, diaphoresis, mydriasis, respiratory distress, muscle tremor and spasms, muscle weakness, rhabdomyolysis, and seizures. Several other effects have been associated with intoxication by synthetic cathinones, including chest pain, myocardial infarction, stroke, disseminated intravascular coagulation, hepatic dysfunction, acute kidney injury, cerebral edema, coma, cardiovascular collapse, and death (Borek and Holstege 2012; Carhart-Harris et al. 2011; Coppola and Mondola 2012a, b; Fröhlich et al. 2011; Mugele et al. 2012).

The liver is a major target for many drugs of abuse, such as amphetamines and cocaine (Andreu et al. 1998; James et al. 2011; Kamijo et al. 2002; Kanel et al. 1990). Clinical evidence has shown that the liver is also a target of MDPV toxicity (Borek and Holstege 2012; Fröhlich et al. 2011; Murray et al. 2012). Based on the close chemical and pharmacological similarities shared by amphetamines and β-keto amphetamines, we hypothesized that cathinone derivatives would cause hepatotoxicity like their amphetamine counterparts. In line with this, our group has recently demonstrated the in vitro hepatotoxic potential of four of the most prevalent synthetic cathinones, namely methylone, MDPV, 4-methylethcathinone (4-MEC) and pentedrone (Araujo et al. 2015), with potencies similar to MDMA. However, specific mechanisms and pathways involved are completely unknown. Various factors may contribute to MDPV-induced liver toxicity, including hyperthermia.

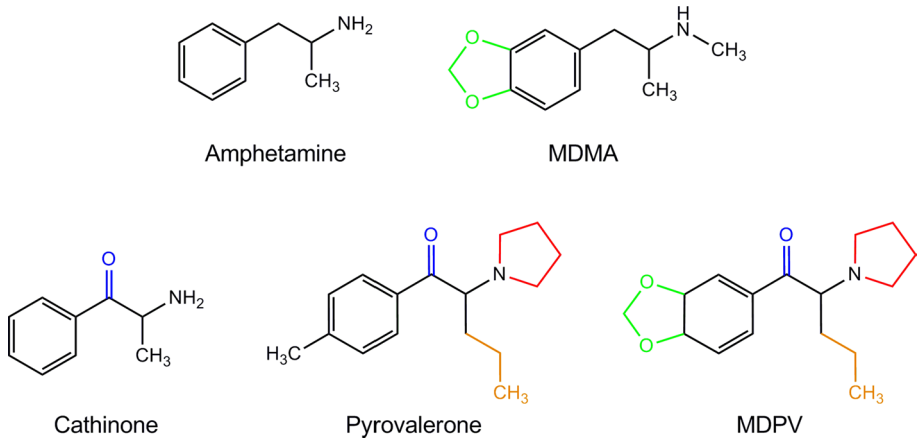

Fig. 1 Chemical structures of amphetamine, MDMA, cathinone, pyrovalerone and MDPV. *MDMA* 3,4-methylenedioxymethamphetamine, *MDPV* 3,4-methylenedioxypyrovalerone (color figure online)

The impairment of thermoregulation elicited by MDPV has also been reported, with significant increases in human body temperature up to values ranging between 39.5 and 41.7 °C (Borek and Holstege 2012; Fröhlich et al. 2011; Kesha et al. 2013; Mugele et al. 2012; Murray et al. 2012). It must be stressed that hyperthermia *per se* is a pro-oxidant aggressive condition (Skibba et al. 1991; Wills et al. 1976), which may render liver cells more vulnerable to oxidants. Accordingly, our group has previously shown that hyperthermia acts synergistically with MDMA-induced toxicity toward freshly isolated mouse hepatocytes (Carvalho et al. 2001) and primary cultured rat hepatocytes (Pontes et al. 2008), but it remains to be investigated whether hyperthermia also exacerbates MDPV toxic responses in vitro. Therefore, the goal of the present study was to provide insights into the mechanisms involved in the toxicity elicited by MDPV in primary cultures of rat hepatocytes under normothermic and hyperthermic conditions (37 and 40.5 °C, respectively). The specific objectives of this study were to (1) evaluate the cytotoxic effect of MDPV in primary rat hepatocytes; (2) elucidate the biochemical mechanisms underlying the cellular injury, namely the generation of ROS and RNS, the interference of MDPV in the homeostasis of glutathione and calcium, and with mitochondrial function; (3) characterize the nature of cell death in MDPV-exposed rat hepatocytes; and (4) investigate the role of hyperthermia in MDPV hepatotoxicity.

Materials and methods

Chemicals

Hank's balanced salt solution (HBSS), heat-inactivated fetal bovine serum (FBS), antibiotic mixture of penicillin/streptomycin (10,000 U/mL/10,000 µg/mL) and fungizone (250 µg/mL) were obtained from GIBCO Invitrogen (Barcelona, Spain). Collagenase from *Clostridium histolyticum* Type IA, bovine serum albumin (BSA), 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), ethylene glycol-bis-(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), Williams' E Medium, dexamethasone, gentamicin sulfate salt, insulin solution from bovine pancreas (10 mg/mL), thiazolyl blue tetrazolium bromide (MTT), sodium pyruvate, β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (β-NADH), 2′,7′-dichlorodihydrofluorescein (DCFH) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), L-glutathione reduced (GSH) and L-glutathione oxidized disodium salt (GSSG), β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (β-NADPH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), adenosine triphosphate (ATP), luciferase from *Photinus pyralis* (firefly) and D-luciferin sodium salt, N-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-pNA, caspase 3 substrate), N-acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide (Ac-IETD-pNA, caspase 8 substrate), and N-acetyl-Leu-Glu-His-Asp-*p*-nitroanilide (Ac-LEHD-pNA, caspase 9 substrate) were purchased from Sigma-Aldrich (St. Louis, MO). Fluo-3 AM, FluoroPure™ grade was obtained from Molecular Probes (Eugene, OR, USA). All other chemicals, of analytical grade, were purchased from Merck (Darmstadt, Germany). MDPV hydrochloride was purchased online from the Sensearomatic website (http://sensearomatic.net, currently unavailable), during March 2013. The salt was fully characterized by mass spectrometry, Nuclear Magnetic Resonance (NMR), and elemental analysis, and purity was greater than 98 %.

Isolation and primary culture of rat hepatocytes

Rat hepatocytes were isolated from male Wistar Han rats (200–250 g) purchased from Charles-River Laboratories (Barcelona, Spain). All experiments were approved by the local ethics committee for the welfare of experimental animals and performed in accordance with national legislation. Surgical procedures were conducted under anesthesia by inhalation of isoflurane, in an isolated system, and carried out between 10.00 and 11.00 a.m. Cells were isolated through a collagenase perfusion, as previously described by our group (Pontes et al. 2008). Briefly, a cannula was inserted in the hepatic portal vein, and the liver was perfused initially with Hank's washing buffer containing BSA and the chelating agent EGTA, followed by a solution of collagenase supplemented by its co-factor calcium. The liver capsule was then gently disrupted in order to release isolated liver cells into a Krebs–Henseleit buffer. The cell suspension was subsequently purified through three cycles of low-speed centrifugations (300 rpm, for 2 min). The final suspension was then incubated with penicillin/streptomycin (500 U/mL/500 µg/mL), at 4 °C, for 30 min. Cell viability was estimated by the trypan blue exclusion test and was always higher than 80 %. A suspension of 500,000 viable cells/mL was cultured in 6- or 96-well plates at approximately 100,000 cells/cm^2, in William's E medium, supplemented with 10 % FBS, 100 U/mL/100 µg/mL penicillin/streptomycin, 5 µg/mL insulin, 50 µM dexamethasone, 100 µg/mL gentamicin and 2.5 µg/mL fungizone, and incubated overnight at 37 °C, with 5 % CO_2, to allow cell adhesion.

Cell viability assays

Hepatocytes were seeded in 96-well plates and exposed to MDPV at a concentration range that includes low-effect

to worst-case approach concentrations (0.2–1.6 mM) in serum-free medium, under normothermic (37 °C) and hyperthermic (40.5 °C) conditions. Cell viability was evaluated after 48 h through the MTT reduction assay and confirmed by the LDH leakage assay, as previously described (Araujo et al. 2015; Barbosa et al. 2014), with minor modifications. For the MTT reduction assay, cells were incubated with a solution of 0.5 mg/mL MTT for 80 min, and the formazan crystals formed through mitochondrial succinate dehydrogenase were dissolved in DMSO and detected at 550 nm in a 96-well plate reader (PowerWaveX; Bio-Tek, Winooski, VT, USA). For measurement of the amount of LDH released into the culture medium, the plates were centrifuged for 10 min at 250*g*, and 50 µL of the incubation medium (dilutions prepared in phosphate buffer: 50 mM KH_2PO_4, pH 7.4) were collected from each well in duplicates into new 96-well plates, to which 200 µL of a 0.21 mM β-NADH solution were added. The kinetic oxidation of β-NADH into β-NAD^+ after adding 25 µL of 22.7 mM sodium pyruvate was monitored at 340 nm in a 96-well absorbance plate reader. Data were normalized to a no-effect (no treatment) and a maximum-effect (lysed with 1 % Triton X-100) controls.

Characterization of hepatotoxicity mechanisms triggered by MDPV

Measurement of intracellular GSH and GSSG levels

Total glutathione (tGSH) and oxidized glutathione (GSSG) contents were determined through the DTNB-GSSG reductase recycling assay (Carvalho et al. 2004). For this purpose, hepatocytes were seeded in 6-well plates and exposed to MDPV, at 37 °C or 40.5 °C. After a 48 h incubation period, cells were rinsed with HBSS without calcium and magnesium, scrapped and precipitated with 5 % perchloric acid for 20 min, at 4 °C. Suspensions were centrifuged at 6000*g*, for 5 min, at 4 °C, and the supernatant was then neutralized with 0.76 M $KHCO_3$, on ice. The samples were spinned at 13,000 rpm, and 100 µL of the supernatants were added to a 96-well plate, followed by 65 µL of a freshly prepared reagent solution of 71.5 mM phosphate buffer with 0.63 mM EDTA containing 0.7 mM NADPH and 4 mM DTNB. Samples were incubated for 15 min at 30 °C in a thermomax 96-well plate reader, and 40 µL of 10 U/mL glutathione reductase was then rapidly added to all samples. The consequent formation of 5-thio-2-nitrobenzoic acid (TNB) was followed for 2 min at 415 nm and compared with a GSH standard curve prepared in 5 % $HClO_4$. For GSSG quantification, precipitated samples were incubated with 2-vinylpyridine for 1 h, at 4 °C, with continuous agitation, in order to derivatizate GSH. GSSG was then quantified as described for tGSH. All data were normalized to total protein content of each sample, measured by the Lowry method, using standard stock solutions of BSA. GSH levels were calculated by subtracting GSSG content from tGSH values as follows: GSH = tGSH − (2 × GSSG).

Measurement of intracellular reactive oxygen and nitrogen species

Intracellular generation of reactive oxygen (ROS) and nitrogen (RNS) species was monitored via DCFH-DA fluorescence assay as previously described (da Silva et al. 2014). DCFH-DA is a cell-permeable fluorogenic probe that readily diffuses into the cells, where it is deacetylated by cellular esterases, and the resultant non-fluorescent DCFH is further oxidized by ROS and RNS to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). For this assay, after overnight cell adhesion in 96-well plates, hepatocytes were pre-incubated with 10 µM DCFH-DA, at 37 °C, protected from light. After 30 min, cells were rinsed with HBSS without calcium and magnesium and incubated with 0.2–1.6 mM MDPV for 48 h, at 37 or 40.5 °C. The fluorescence was then recorded on a microplate reader (Synergy HTX Multi-Mode Reader; Bio-Tek, Winooski, VT, USA), set to 485 nm excitation and 530 m emission. Results were normalized to negative controls (no treatment) and calculated as fold increase over control. No interference with the probe was noted at any tested concentration of MDPV, as determined by incubation for 48 h with DCFH in the absence of cells (data not shown).

Measurement of intracellular ATP levels

The ATP bioluminescence assay is based on the emission of light from the reaction of ATP and luciferin, catalyzed by luciferase and was performed as previously described (Valente et al. 2012). After exposure to MDPV, cells were rinsed and treated as described above for tGSH measurement. After neutralization, samples were spinned, and 100 µL of the supernatants were added to 96-well opaque plates with 100 µL of luciferin–luciferase assay solution [final concentrations: 0.15 mM luciferin, 30,000 light units luciferase, 50 mM glycine, 10 mM MgSO4, 1 mM Tris, 0.55 mM EDTA, 1 % BSA (pH 7.6)]. ATP calibration curve was obtained with standard stock solutions of ATP prepared in 5 % $HClO_4$. Results were normalized to total protein, measured by the Lowry method.

Quantification of intracellular free calcium (Ca^{2+}) levels by flow cytometry

Fluo3-AM is a sensitive fluorochrome that is enzymatically hydrolyzed by cellular esterases to give Fluo3, which exhibits an increase in fluorescence upon binding with

calcium. For this assay, cells from six-well plates exposed to 0.2–1.6 mM MDPV for 48 h were collected after trypsinization with 0.05 % trypsin/EDTA, centrifuged (250*g*, 5 min, 4 °C), and rinsed with HBSS without calcium and magnesium. After further centrifugation, the supernatant was discarded and samples were incubated for 30 min, with continuous agitation, at 37 °C, with 50 µL of a 10 µM Fluo3-AM solution prepared in serum-free Williams' E Medium without phenol red. Following incubation, the cells were washed and resuspended with HBSS with calcium and magnesium and kept on ice until further analysis. Samples were analyzed in a BD Accuri™ C6 flow cytometer (BD Biosciences, CA, USA), with the FCS Express™ analysis software. The green fluorescence of Fluo3 was detected through a 533/30 nm filter (FL1 detector). The DNA of non-viable cells was stained with 5 µg/mL propidium iodide (PI), which has a maximum fluorescence emission at 617 nm, and the green fluorescence from PI positive cells was excluded from the analysis. At least 20,000 viable cells were analyzed per sample. There was no contribution of autofluorescence, as determined by the analysis of treated cells in the absence of Fluo3-AM (data not shown). Data were normalized to controls without treatment.

Characterization of death pathways triggered by MDPV

Measurement of caspase 3, 8 and 9 activity

The activities of caspase 3, 8 and 9 were determined in cytoplasmatic fractions of hepatocytes after exposure to 0.2–1.6 mM MDPV for 48 h, as previously described (Capela et al. 2007), with adaptations. This assay was based on the hydrolysis of the peptide substrates for each caspase, namely Ac-DEVD-pNA, Ac-IETD-pNA and Ac-LEHD-pNA, for caspase 3, 8 and 9, respectively. This reaction results in the release of the p-nitroaniline moiety, with peak absorbance at 405 nm. For each condition, three wells of a 6-well plate were rinsed with HBSS without calcium and magnesium, and pellets were collected into new tubes with 225 µL of lysis buffer [final concentrations: 50 mM HEPES, 1 mM DTT, 0.1 mM EDTA, 0.1 % CHAPS (pH 7.4)]. Samples were incubated for 5 min on ice, centrifuged (16,000*g*, 10 min, 4 °C), and the cell lysate transferred into new tubes. Two hundred microliters of assay buffer [final concentrations: 100 mM NaCl, 50 mM HEPES, 10 mM DTT, 1 mM EDTA, 10 % glycerol, 0.1 % CHAPS (pH 7.5)] were added to 50 µL of lysate in 96-well plates, followed by 5 µL of colorimetric substrate (16 µM Ac-DEVD-pNA, 200 µM Ac-IETD-pNA or 200 µM Ac-LEHD-pNA). After 24 h at 37 °C, absorbance was determined at 405 nm in a 96-well absorbance plate reader. Protein content in cell lysate was measured using the Bio-Rad RC DC protein assay kit (Hercules, CA, USA), with BSA as the standard.

Hoechst 33342/propidium iodide fluorescent staining

Apoptotic hepatocytes were identified based on chromatin morphology, as previously described (Valente et al. 2012), using Hoechst 33342, a cell-permeant nuclear counterstain that emits blue fluorescence when bound to DNA, and PI, a membrane impermeant nuclear dye that emits red fluorescence only in dead cells. Briefly, primary rat hepatocytes were seeded into six-well plates and exposed to MDPV (0.2–1.6 mM) for 48 h, at 37 or 40.5 °C. After washing with HBSS without calcium and magnesium, cells were incubated with 50 µM PI for 15 min, rinsed again, and fixed with a 4 % solution of p-formaldehyde for 20 min. After washing, cells were incubated with a 5 µg/mL Hoechst 33342 solution for 5 min and observed under a fluorescent microscope.

Statistical analysis

Data were obtained from at least five independent experiments, performed in duplicate or triplicate, and are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using the GraphPad Prism 6 (version 6.01) for Windows. Multiple comparisons within the two variables (concentration and temperature) were performed through one-way ANOVA analysis, followed by Fisher's LSD post hoc test. Significance was accepted for $p < 0.05$.

Results

MDPV induces cell death in a concentration-dependent manner, exacerbated in hyperthermic conditions

The data presented in Fig. 2 show a concentration-dependent increase in cytotoxicity, as determined by the MTT reduction assay, in primary rat hepatocytes exposed to MDPV at 37 °C. This effect was significant ($p < 0.01$ vs. control), even at the lowest concentration tested (0.2 mM). Since MDPV is a ketone, and thus, a possible redox electron donor that may interfere with redox-based tests like the MTT assay (den Hollander et al. 2014), we additionally performed the interference-free and unbiased LDH leakage assay, under the same experimental conditions, and the obtained results are shown in Fig. 3. Data from this assay corroborate the results from the MTT test, showing cell death in identical magnitude and significance, which suggests that there is no substantial reduction of the tetrazolium dye by MDPV under our in vitro conditions.

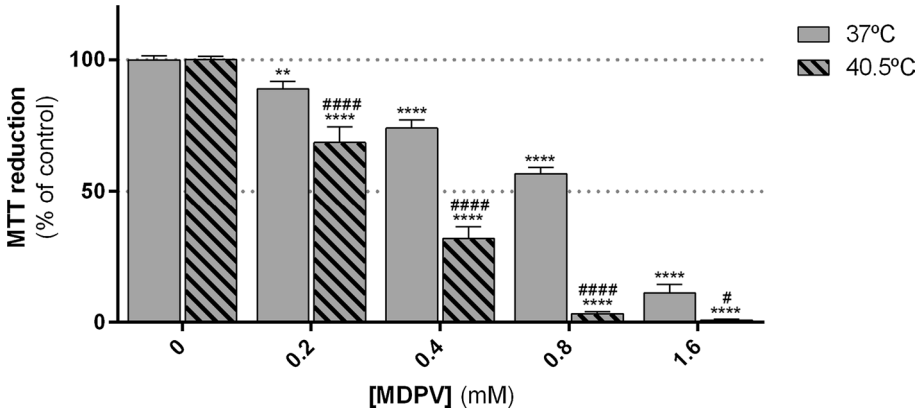

Fig. 2 Effects of MDPV on MTT reduction in primary cultured rat hepatocytes. Hepatocytes were exposed for 48 h, at 37 or 40.5 °C, to 0.2–1.6 mM MDPV. Results are presented as mean ± SEM from at least five independent experiments, performed in triplicate. $^{**}p < 0.01$, $^{****}p < 0.0001$ versus control. $^{\#}p < 0.05$, $^{\#\#\#\#}p < 0.0001$ versus normothermia

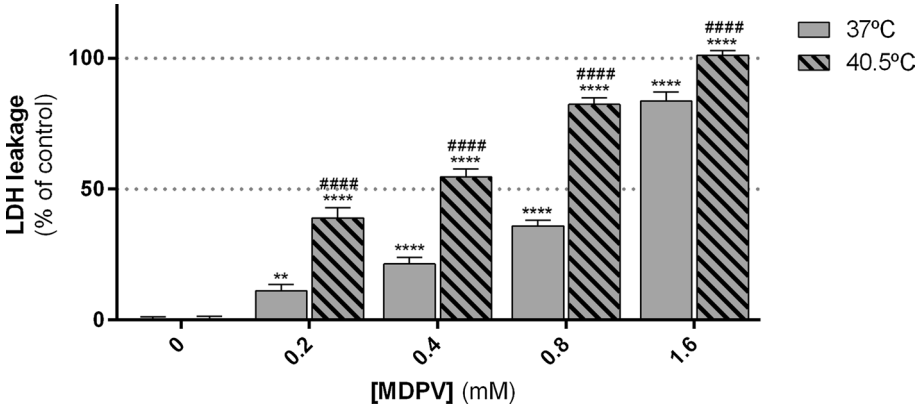

Fig. 3 Effects of MDPV on LDH leakage in primary cultured rat hepatocytes. Hepatocytes were exposed for 48 h, at 37 or 40.5 °C, to 0.2–1.6 mM MDPV. Results are presented as mean ± SEM from six independent experiments, performed in triplicate. $^{**}p < 0.01$, $^{****}p < 0.0001$ versus control. $^{\#\#\#\#}p < 0.0001$ versus normothermia

Noteworthy, a rise in incubation temperature from 37 to 40.5 °C greatly exacerbated cell death induced by MDPV, for all concentration range ($p < 0.0001$ vs. normothermia). For instance, as observed in the MTT assay, cell death induced by 0.2 mM MDPV increased from 11.1 ± 2.9 % in normothermic conditions up to 31.4 ± 5.9 % in hyperthermic conditions.

MDPV triggers a marked GSH depletion and ROS and RNS formation in primary rat hepatocytes, exacerbated in hyperthermic conditions

Since oxidative stress is a well-known mechanism involved in the hepatotoxicity of non-keto amphetamines (Carvalho et al. 2012), we started to investigate whether this mechanism is also shared by MDPV. Glutathione provides a major cell antioxidant defense against reactive species formation and ensuing oxidative damage. Therefore, the intracellular content of GSH and GSSG was measured after exposure of primary rat hepatocytes to MDPV (0.2–1.6 mM), at 37 and 40.5 °C. As depicted in Fig. 4a, at 37 °C, MDPV caused a significant concentration-dependent decline in GSH levels, with a decrease from 39.1 ± 3.2 nmol/mg of protein in control cells to 15.4 ± 2.9 nmol/mg of protein in cells treated with 1.6 mM MDPV. GSH depletion was clearly more accentuated under hyperthermic conditions. In fact, the highest concentration studied (1.6 mM) caused an almost complete depletion of GSH levels (0.4 ± 0.1 nmol/mg of protein) at 40.5 °C ($p < 0.0001$ vs. control and normothermia). An important finding was that, under normothermic conditions, the GSH depletion induced by MDPV was not accompanied by glutathione oxidation (Fig. 4b). However, it is noteworthy that hyperthermia, by itself, elicited a significant decline in basal GSH levels of control cells of approximately 36 % ($p < 0.0001$), as well as an increase of nearly 100 % in GSSG levels ($p < 0.05$).

The involvement of oxidative stress in MDPV-induced toxicity was further assessed through the measurement of ROS and RNS generation at the same conditions. The levels of reactive species following 48 h exposure with MDPV at normothermic and hyperthermic conditions are shown in the Fig. 5. At 37 °C, MDPV increased the formation of reactive species after exposure to the highest concentration studied (1.6 mM), with a 1.50 ± 0.04-fold increase over control cells ($p < 0.0001$ vs. control). At 40.5 °C, the levels of ROS and RNS were prominently increased in a concentration-dependent manner following incubation with MDPV, with a 1.14 ± 0.03, 1.29 ± 0.03 and 1.65 ± 0.06-fold increase in cells treated with 0.4, 0.8 and 1.6 mM MDPV, respectively.

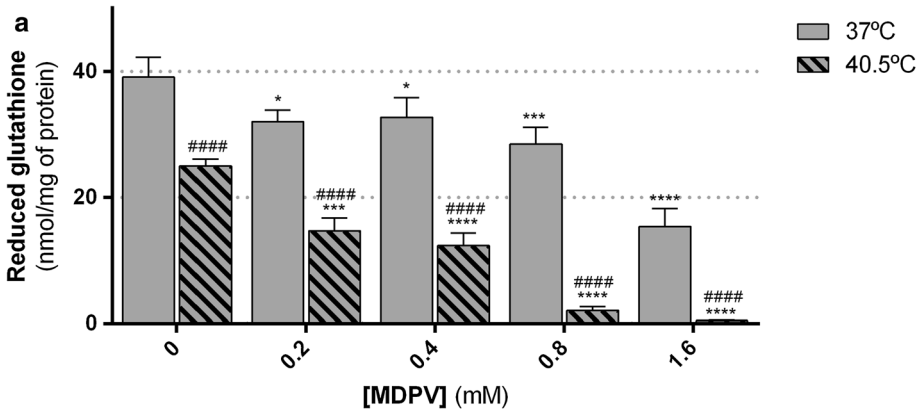

Fig. 4 Effects of MDPV on **a** GSH and **b** GSSG levels in primary cultured rat hepatocytes. Hepatocytes were exposed for 48 h, at 37 or 40.5 °C, to 0.2–1.6 mM MDPV. Results are presented as mean ± SEM from at least five independent experiments, performed in triplicate. Please note that different scales on the *y*-axis are used in **a** and **b**. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ versus control. $^{\#}p < 0.05$, $^{\#\#\#\#}p < 0.0001$ versus normothermia

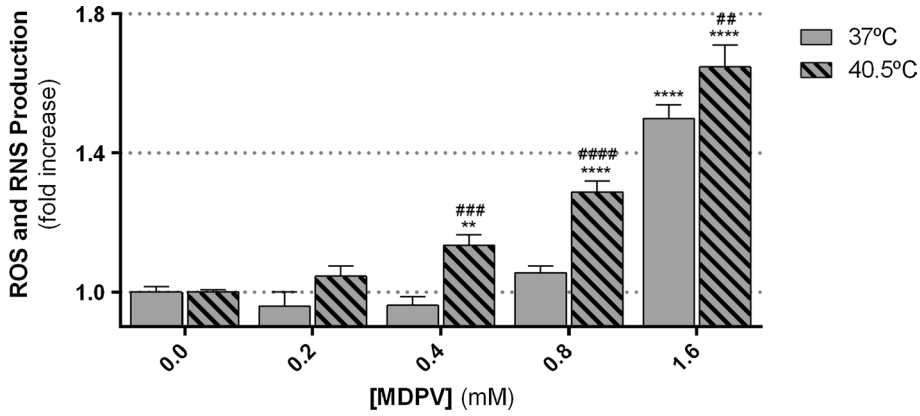

Fig. 5 Effects of MDPV on ROS and RNS production in primary cultured rat hepatocytes. Hepatocytes were exposed for 48 h, at 37 or 40.5 °C, to 0.2–1.6 mM MDPV. Results are presented as mean ± SEM from six independent experiments, performed in triplicate. $^{**}p < 0.01$, $^{****}p < 0.0001$ versus control. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ versus normothermia

MDPV hampers the hepatocellular energetic status and Ca^{2+} homeostasis, especially at high concentrations and in hyperthermic conditions

To better understand the potential mechanisms involved in MDPV-induced toxicity in primary rat hepatocytes, we investigated whether mitochondrial bioenergetic function or calcium homeostasis are affected. Figure 6 depicts the intracellular levels of ATP in MDPV-treated cells at both incubation temperatures herein studied (37 and 40.5 °C). At 37 °C, it was observed a pronounced decline (from 23.6 ± 4.2 to 8.3 ± 1.6 nmol/mg of protein) in intracellular ATP levels in cells exposed to 1.6 mM MDPV ($p < 0.001$ vs. control), with no significant changes detected at lower concentrations. The increase in incubation temperature to 40.5 °C prompted an abrupt energy depletion in hepatocytes treated with 0.8 mM and 1.6 mM MDPV (from 22.7 ± 1.9 in control cells to 1.8 ± 0.3 and 0.3 ± 0.1 nmol/mg, respectively), and to a less extent with 0.4 mM MDPV. Moreover, as shown in Fig. 7, MDPV at these two highest concentrations also affects the intracellular Ca^{2+} homeostasis, whereas no significant changes were noticed at lower concentrations. No statistically significant differences were detected between normo- and hyperthermic conditions.

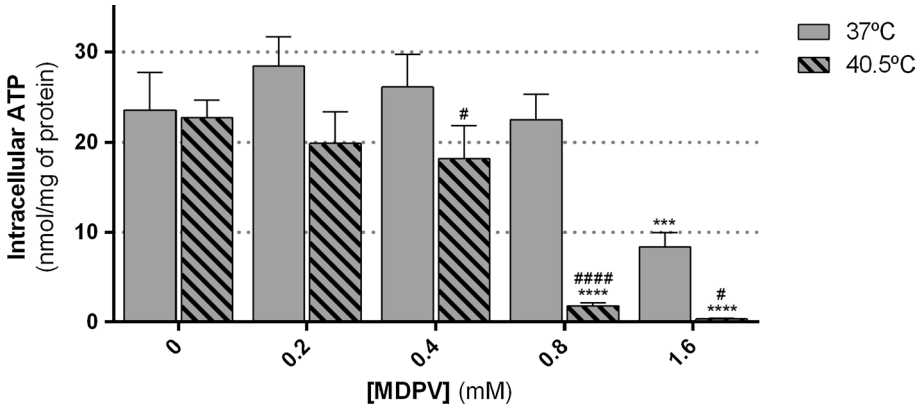

Fig. 6 Effects of MDPV on ATP levels in primary cultured rat hepatocytes. Hepatocytes were exposed for 48 h, at 37 or 40.5 °C, to 0.2–1.6 mM MDPV. Results are presented as mean ± SEM from at least five independent experiments, performed in duplicate. ***$p < 0.001$, ****$p < 0.0001$ versus control. $^{\#}p < 0.05$, $^{\#\#\#\#}p < 0.0001$ versus normothermia

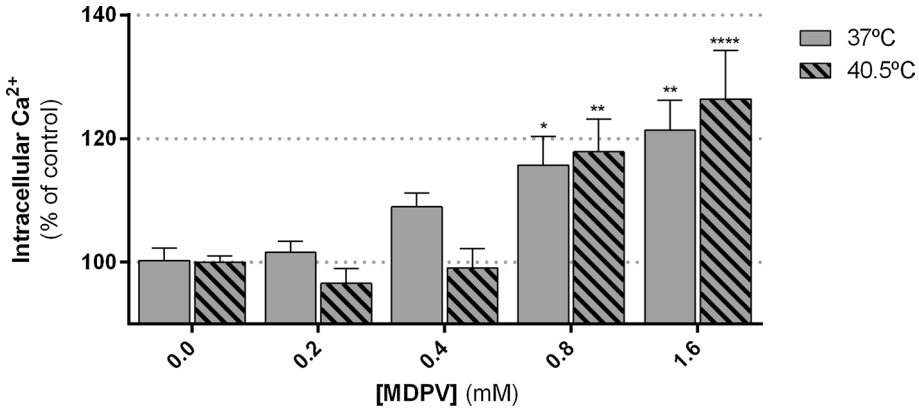

Fig. 7 Effects of MDPV on intracellular Ca^{2+} levels in primary cultured rat hepatocytes. Hepatocytes were exposed for 48 h, at 37 or 40.5 °C, to 0.2–1.6 mM MDPV. Results are presented as mean ± SEM from at least five independent experiments, performed in duplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control

MDPV induces primarily apoptotic cell death in normothermia, but shifts to necrosis under hyperthermic conditions

To determine whether MDPV-induced hepatotoxicity involves the initiation of apoptotic cell death mechanisms, two different methodological approaches were followed: (1) fluorescence microscopy analysis after staining with Hoechst 33342/PI and (2) measurement of caspase 3, 8 and 9 activity. Nuclear morphological changes of MDPV-treated primary rat hepatocytes were examined using Hoechst 33342, a membrane permeable blue dye for DNA labeling, and PI, a fluorescent red dye that intercalates into double-stranded nucleic acid of late apoptotic or necrotic cells. Figure 8 shows representative Hoechst 33342/PI fluorescence photomicrographs of cultured cells treated with and without MDPV at two different incubation temperatures. In control cells, at 37 °C, nuclei appeared with regular contours and were round and large in size, whereas, at 40.5 °C, it was possible to identify early apoptotic events (pyknotic nuclei formation without PI label, green arrows). In MDPV-treated cells, under normothermic conditions, the pyknotic nuclei were visible even at the lowest MDPV concentration tested, as well as late apoptotic cells (red condensed nuclei). On the other hand, necrotic cells (red large nuclei) were only observable in 0.8 and 1.6 mM MDPV-exposed cells. In contrast, a rise in incubation temperature to 40.5 °C clearly favoured necrosis in hepatocytes treated with MDPV, as red large nuclei are seen under exposure to concentrations as low as 0.2 mM.

Since caspase activation is one of the main events leading to apoptosis, we additionally confirmed apoptotic cell death by evaluating the activation of caspases 8 (extrinsic pathway), 9 (intrinsic pathway), and 3 (effector caspase) at 37 and 40.5 °C. As can be seen in Fig. 9, at 37 °C, 1.6 mM MDPV significantly increased caspase 3, 8 and 9 activities in primary rat hepatocytes about 118, 55 and 54 % over control values, respectively ($p < 0.0001$ vs. control). Noteworthy, the increase in incubation temperature to 40.5 °C triggers caspase activation at lower concentrations. Under hyperthermia, a peak in caspase activation was achieved at 0.8 mM MDPV, with increases up to 156, 64 and 50 % over control values ($p < 0.0001$ vs. control), and further reduced at 1.6 mM MDPV to values near to controls for caspase 8 and 9 ($p > 0.05$ vs. control, $p < 0.001$ vs. normothermia), respectively.

Discussion

MDPV structure is closely related to amphetamines that are known to be hepatotoxic in humans (Carvalho et al. 2012).

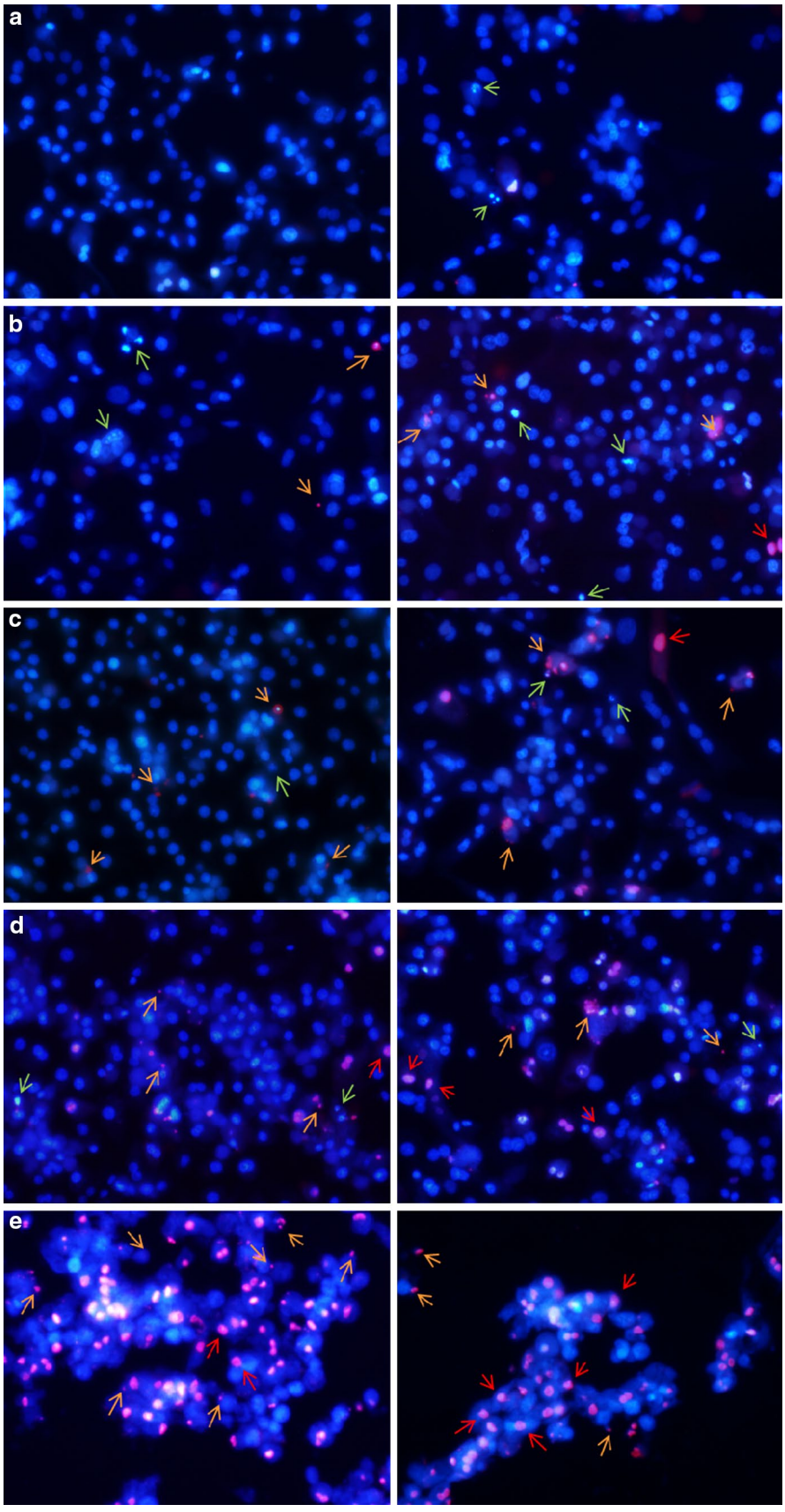

Fig. 8 Representative fluorescence microscopy images of Hoechst 33342/PI staining. Hepatocytes were exposed for 48 h, at (*left*) 37 or (*right*) 40.5 °C, to **a** 0, **b** 0.2, **c** 0.4, **d** 0.8 or **e** 1.6 mM MDPV. *Green arrows* indicate early apoptotic cells, *orange arrows* indicate late apoptotic cells and *red arrows* indicate necrotic cells. Original magnification, ×200 (color figure online)

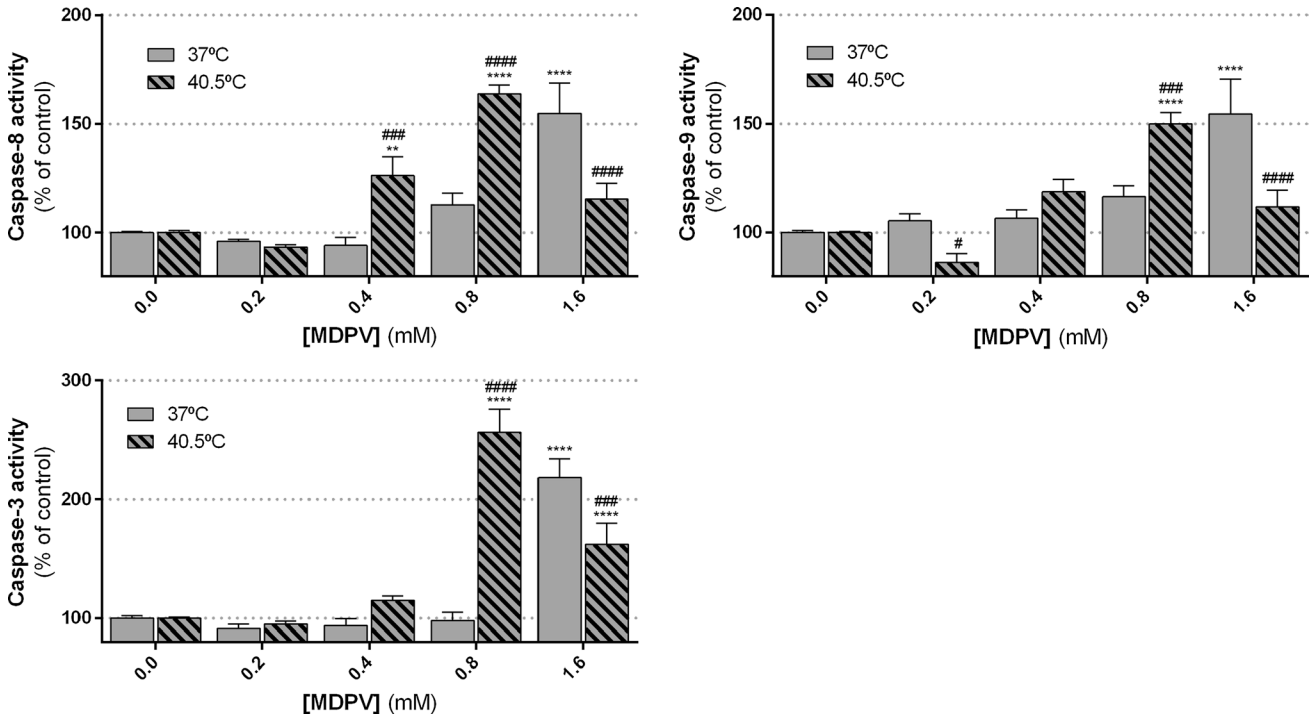

Fig. 9 Effects of MDPV on the activities of caspases 3, 8 and 9 in primary cultured rat hepatocytes. Hepatocytes were exposed for 48 h, at 37 or 40.5 °C, to 0.2–1.6 mM MDPV. Results are presented as mean ± SEM from at least five independent experiments, performed in duplicate. $^{**}p < 0.01$, $^{****}p < 0.0001$ versus control. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ versus normothermia

Therefore, toxicological studies of cathinone designer drugs toward liver cells are of particular interest. In this study, we first tested and confirmed the ability of the synthetic cathinone MDPV to induce cytotoxicity in primary rat hepatocytes and contributed to the elucidation of the mechanisms involved in MDPV-induced hepatocellular injury at normothermia and hyperthermia. We showed that MDPV-induced cell death in a concentration- and temperature-dependent manner, through two different cytotoxicity tests, namely by inhibiting the reduction of MTT and promoting LDH leakage (Figs. 2, 3, respectively). These findings are consistent with our previous study showing the hepatotoxic potential of four of the most widely abused cathinone derivatives, including MDPV (Araujo et al. 2015).

Several mechanisms induced by drugs of abuse may lead to liver damage. Among them, oxidative stress is thought to play a major role in the hepatocellular damage consequent to the abuse of amphetamines (Beitia et al. 2000; Carvalho et al. 2010). Due to structural similarities to their non-keto congeners, we hypothesize that synthetic cathinones may undergo similar mechanisms of toxicity. In fact, our results suggest that MDPV and/or its intermediate metabolites, similarly to MDMA (Cerretani et al. 2011), cause oxidative stress in the liver, as evidenced by substantial depletion in the intracellular levels of GSH (Fig. 4a), which is an endogenous antioxidant known to play a crucial role in cell survival, and increased formation of ROS and NOS (Fig. 5). Importantly, we found that MDPV-induced GSH depletion was not accompanied by an increase in GSSG levels. This result might be explained by ATP-dependent export of the formed GSSG into the extracellular space (Leier et al. 1996). However, in this experiment, GSH depletion seems to be primarily related to the formation of conjugates of MDPV with GSH, rather than a direct effect of reactive species, since the rise in ROS and RNS production was only observed in cells exposed to MDPV at high concentrations, where a severe GSH depletion in the cytosolic GSH pool had already occurred. In accordance, metabolic activation to a reactive metabolite capable of conjugating with GSH has been recently demonstrated for MDPV in vitro using human liver microsomes (Meyer et al. 2014; Strano-Rossi et al. 2010), and in vivo in rats and humans (Meyer et al. 2010) and, most certainly, occurs in our in vitro model. Therefore, MDPV metabolism and the ensuing depletion of intracellular GSH levels lowers the capacity of the cells to scavenge reactive species, enhancing oxidative stress, disturb cellular function and, ultimately, may lead to cell death.

Moreover, it must be highlighted that hepatic metabolism of amphetamines that contain a 3,4-methylenedioxy ring is an important source of ROS and other chemically

reactive compounds (Carvalho et al. 2010, 2012). This methylenedioxy group is also present in MDPV but, presently, it is not clear if any MDPV metabolites formed in cells are capable to undergo oxidation into reactive orthoquinones that enter redox cycling with subsequent production of ROS and RNS, as has been thoroughly described for MDMA (Carvalho et al. 2010). Therefore, it is still unknown whether the generation of reactive species in hepatocytes exposed to MDPV depends either on disturbance of the mitochondrial respiratory chain (well-known as the major cellular source of ROS generation) or MDPV metabolism, or even a combination of both.

There is a complex cross-talk between oxidative stress, Ca^{2+} homeostasis and mitochondrial function. As the main organelles responsible for ATP production, mitochondria are the core of cell energy supply and participate actively in the sequestration of free Ca^{2+} (Brookes et al. 2004). Our results showed that MDPV, at a high concentration (1.6 mM), triggered a drastic ATP depletion (Fig. 6). The lack of ATP might lead to a less efficient calcium control and mitochondrial electron chain regulation, as can be seen with the increase in intracellular Ca^{2+} content in primary rat hepatocytes treated with 1.6 mM MDPV for 48 h at 37 °C (Fig. 7), thus indicating that the mitochondrial function and calcium homeostasis are severely impaired by MDPV at high concentrations. This decreases in ATP levels occurred at a concentration where GSH depletion was almost complete, with overproduction of ROS and RNS, suggesting that oxidative stress may be involved in the disturbance of mitochondrial functions. On the other hand, it is possible that MDPV and/or its metabolites may inhibit the mitochondrial function by directly interacting with mitochondrial proteins. Further studies are necessary to determine the mechanisms of mitochondrial impairment induced by MDPV.

As center of cell death control, mitochondria undergo extensive membrane integrity and permeability changes prior to cell death itself (Tait and Green 2010). The mitochondrial permeability transition pore (MPTP), a large nonspecific conductance channel assembled from proteins in the inner and outer mitochondrial membranes, appears to play a key role in triggering of an intrinsic apoptotic pathway, and Ca^{2+} is also in control of the process of MPTP opening (Brookes et al. 2004; Halestrap 2009). In fact, Baumgartner et al. (2009) showed that the elevation of mitochondrial Ca^{2+} is the decisive factor in determining whether cells undergo oxidative stress-induced apoptosis. When there is an overload of Ca^{2+} in the mitochondrial matrix, the MPTP opens and allows free passage of ions and solutes under 1500 Da across the mitochondrial inner membrane. This effect has two outcomes: entering of protons that lead to uncoupling of the oxidative phosphorylation, and consequent impairment of ATP synthesis (Halestrap 2006, 2009); and swelling of the mitochondria matrix, with subsequent rupture of the outer membrane, and nonspecific release of pro-apoptotic proteins into the cytosol, including cytochrome *c* (Tait and Green 2010). Alternatively, permeabilization of the outer mitochondrial membrane can be achieved by pore formation by pro-apoptotic Bcl-2 family proteins (Bax and Bak) (Tsujimoto 2003). Once in the cytosol, cytochrome *c* triggers a complex signaling cascade that ultimately ends in cell death through apoptosis, with activation of caspase 9, which further cleaves and activates the executioner caspases 3 and 7 that, in turn, cleave intracellular substrates, causing the morphological and biochemical changes observed in the process of apoptosis. These caspases may also enter the mitochondrial intermembrane space, disrupting the respiratory chain, consequently leading to a decline in ATP synthesis and increase in ROS production, which further amplifies apoptosis (McIlwain et al. 2013; Tait and Green 2010). Besides this intrinsic pathway, xenobiotics may also induce mitochondria-independent apoptotic pathways. This extrinsic process is initiated by the binding of ligands to death receptors located in the cell membrane, leading to the activation of caspase 8, which directly initiates apoptosis by activating the executioner caspases, or indirectly activating the intrinsic apoptotic pathway (McIlwain et al. 2013).

In the present study, it was demonstrated that MDPV-treated primary rat hepatocytes stained with Hoechst 33342/PI, in the absence of heat stress (at 37 °C), preferentially exhibited death with apoptotic characteristics, with an increase in early apoptotic cells, evident at concentrations as low as 0.2 mM (Fig. 8). Typical nuclear morphology changes of cells undergoing apoptosis, such as condensation and fragmentation of nuclei chromatin, were observed in cells exposed to MDPV at lower concentrations, while necrotic events were only evident at the highest concentrations (0.8 and 1.6 mM). This result is indicative of a bimodal cell death, with apoptosis at lower concentrations and necrosis at higher concentrations. As previously referred in the LDH data (Fig. 3), the supernatants of hepatocytes exposed to MDPV (0.2–1.6 mM) contained significantly higher amounts of LDH all over the experimental period, indicating a loss of integrity of the plasma membrane. It is important to stress at this point that the LDH release assay does not distinguish between primary necrosis and secondary necrosis as a consequence of apoptotic cell death. Based on our findings, any detected LDH release in cells exposed to low concentrations of MDPV is more likely due to secondary necrosis from late apoptotic cells, as no signs of primary necrosis were observed in cells exposed to lower concentrations of the tested drug. Moreover, decreases in MTT reduction, a marker of mitochondrial dysfunction, under the same experimental conditions, further supports an apoptotic cell death mechanism.

Additionally, our data on caspase activity, under normothermic conditions, is in agreement with the induction of apoptosis, though only statistically significant at the highest concentration of MDPV tested. After a 48 h period of incubation, MDPV was able to induce both intrinsic (caspase 9) and extrinsic (caspase 8) apoptotic pathways in primary cultures of rat hepatocytes, which was accompanied by a significant activation of the end-line effector caspase common to both pathways, caspase 3. The occurrence of apoptotic events in the liver was also demonstrated in rats administered with MDMA (Cerretani et al. 2011) and recently described in neonatal mouse brain following MDPV administration (Adam et al. 2014).

Hyperthermia is a toxicological effect that has been associated with the abuse of different cathinone derivatives, particularly MDPV (Borek and Holstege 2012; Kesha et al. 2013; Penders et al. 2012). Although it was already demonstrated that hyperthermia potentiates the toxicity induced by amphetamines in several study models including primary hepatocytes (Carvalho et al. 1997, 2001; Pontes et al. 2008), neuronal cells (Capela et al. 2006) and skeletal muscle (Duarte et al. 1999), it remained unknown whether hyperthermia also increases hepatotoxic responses of the bk-amphetamines analogues. The present study provides irrefutable evidence of the potentiation of MDPV toxicity by hyperthermia as a dramatic decrease in cell viability (Figs. 2, 3), as well as an intensification of all tested oxidative stress endpoints were observed in cells exposed to the drug at 40.5 °C. Hyperthermic conditions heightened MDPV-induced depletion of GSH and formation of ROS and RNS (Figs. 4a, 5, respectively). It is worth to note that the observed potentiation of MDPV-induced GSH depletion by hyperthermia was not due to an increase of GSSG formation. There is compelling evidence in the literature that hyperthermia, *per se*, stimulates a pro-oxidant state in the liver, with the depletion of GSH as a probable initiating event (Skibba et al. 1989, 1991). In accordance, our data showed a decline of intracellular GSH levels in control hepatocytes at 40.5 °C (Fig. 4a), as well as a significant rise in GSSG levels (Fig. 4b). This loss of antioxidant defenses may be in the origin of the intensification of MDPV toxic effects under hyperthermia. The rise in temperature also increased mitochondrial energetic impairment in cells exposed to high MDPV concentrations, leading to a nearly complete ATP depletion (Fig. 6), followed by increased intracellular calcium concentrations (Fig. 7).

Moreover, our data suggest that the increment of 3.5 °C increased cell vulnerability toward necrosis, which advocates a change in the mode of cell death, with the increase in temperature, from apoptosis at physiological temperature, to necrosis under hyperthermic conditions. These findings are in agreement with the ATP levels found in cells. In fact, since apoptosis is an energy-consuming process, requiring sufficient ATP to occur, the profound ATP depletion attained in cells treated with MDPV (0.4–1.6 mM) at 40.5 °C forces the cell death mechanisms to shift from apoptosis to necrosis. Our results show that hyperthermia hastened activation of apoptotic cell death in MDPV-exposed cells, as evidenced by caspase activation at lower concentrations (Fig. 9), as well as triggered primary necrotic cell death in rat hepatocytes (Fig. 8). These data are corroborated by the observed decline in caspase activity in cells exposed to 1.6 mM MDPV at 40.5 °C, at a concentration where ATP levels are virtually nonexistent and necrosis ensues. The favouring of a necrotic cell death mechanism over apoptosis under hyperthermic conditions was also described in primary cultured rat hepatocytes (Pontes et al. 2008) and HepG2 cells exposed to MDMA (da Silva et al. 2013). Considering that the higher temperature used in our experiment (40.5 °C) matches the body temperatures that recreational abusers may attain after MDPV consumption (Borek and Holstege 2012; Fröhlich et al. 2011; Kesha et al. 2013; Mugele et al. 2012; Murray et al. 2012), our results suggests that MDPV-induced thermoregulation impairment most certainly contributes to increased liver damage.

Finally, it could be argued that the observed hepatotoxic effects occur at relatively high concentrations of the tested drug when compared to the low micromolar concentrations commonly found in MDPV users or in blood of fatal intoxication victims (Kesha et al. 2013; Marinetti and Antonides 2013; Murray et al. 2012; Wyman et al. 2013). However, similar to other drugs like MDMA, whose levels were found to be up to 18 times higher in the liver (De Letter et al. 2006; Garcıa-Repetto et al. 2003), the drug concentrations to which hepatocytes are actually exposed may be much higher than those found in blood. Moreover, it must be noted that we used the same concentration range typically used in in vitro toxicity studies with amphetamine-type drugs (Beitia et al. 1999; Capela et al. 2006; Carvalho et al. 2004; da Silva et al. 2014; Downey et al. 2014; Pontes et al. 2008). The results from these experiments should be viewed as a way of gaining a better understanding of mechanisms that may be involved in the in vivo effects of MDPV.

Concluding remarks

The data presented in this study strongly suggest that MDPV toxicity in primary cultured rat hepatocytes is mediated by oxidative stress, subsequent to marked GSH depletion, followed by a triangle of events that includes increased ROS and RNS accumulation, mitochondrial dysfunction and perturbation of intracellular Ca^{2+} homeostasis, ultimately leading to apoptotic (for lower concentrations)

and necrotic (for higher concentrations) cell death. Both intrinsic (caspase 9 activation) and extrinsic (caspase 8 activation) apoptotic pathways are stimulated, with subsequent activation of the common effector caspase 3. Furthermore, our results clearly indicate that hyperthermia boosts the oxidative stress induced by MDPV in primary rat hepatocytes that ultimately leads to extensive cell death mainly by necrosis. These results suggest that the rise in body temperature after MDPV intake most certainly contributes to the hepatotoxicity that has been reported in humans. Also of great importance, we evidenced the similarities between the toxic events elicited by the MDPV and amphetamines such as MDMA, thus suggesting a correspondence in the mechanism of toxicity of these drugs.

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Manuscript III. Neurotoxicity of β-keto amphetamines: deathly mechanisms elicited by methylone and MDPV in human dopaminergic SH-SY5Y cells

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Neurotoxicity of β-Keto Amphetamines: Deathly Mechanisms Elicited by Methylone and MDPV in Human Dopaminergic SH-SY5Y Cells

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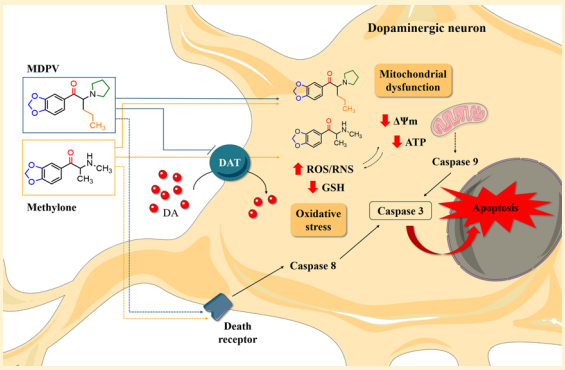
Supporting Information

ABSTRACT: Synthetic cathinones (β-keto amphetamines) act as potent CNS stimulants similarly to classical amphetamines, which raise concerns about their potential neurotoxic effects. The present in vitro study aimed to explore and compare the mechanisms underlying the neurotoxicity of two commonly abused cathinone derivatives, 3,4-methylenedioxymethcathinone (methylone) and 3,4-methylenedioxypyrovalerone (MDPV), with those of 3,4-methylenedioxymethamphetamine (MDMA), using undifferentiated and differentiated SH-SY5Y cells. Following a 24 h exposure period, methylone and MDPV induced loss of cell viability in a concentration-dependent manner, in the following order of potency: MDPV ≈ MDMA > methylone. Dopaminergic differentiated cells evidenced higher sensitivity to the neurotoxic effects of both cathinones and MDMA than the undifferentiated ones, but this effect was not inhibited by the DAT inhibitor GBR 12909. Intracellular oxidative stress mediated by methylone and MDPV was demonstrated by the increase in reactive oxygen and nitrogen species (ROS and RNS) production, depletion of intracellular reduced glutathione and increased oxidized glutathione levels. All three drugs elicited mitochondrial impairment, characterized by the mitochondrial membrane potential (Δψm) dissipation and intracellular ATP depletion. Apoptosis was found to be a common mechanism of cell death induced by methylone and MDPV, with evident chromatin condensation and formation of pyknotic nuclei, and activation of caspases 3, 8, and 9. In conclusion, the present data shows that oxidative stress and mitochondrial dysfunction play a role in cathinones-induced neuronal damage, ultimately leading to cell death by apoptosis.



KEYWORDS: *β-Keto amphetamines, synthetic cathinones, neurotoxicity, oxidative stress, mitochondrial impairment, apoptosis*

INTRODUCTION

Synthetic cathinones emerged this century as a novel class of recreational designer drugs and have grown to be popular drugs of abuse worldwide, with special incidence among young adults.[1] These substances, also known as β-keto amphetamines, are chemically related to classical amphetamines, bearing a ketone group at the β-position of the side chain,[2] and act essentially as stimulants of the central nervous system (CNS).[3] Synthetic cathinones are currently the second largest group of new psychoactive substances (NPS) monitored globally,[4,5] with 103 different derivatives identified so far.[5] Methylone and 3,4-methylenedioxypyrovalerone (MDPV) are two of the most commonly abused derivatives worldwide,[6] and belong to different chemical subgroups of cathinones: methylone is the β-keto analogue of 3,4-methylenedioxymethamphetamine (MDMA), whereas MDPV is a pyrrolidinophenone analogue, containing a pyrrolidine group besides the 3,4-methylenedioxy ring.[7] Similarly to long-standing drugs of abuse such as amphetamines and cocaine, the psychostimulant effects of β-keto amphetamines arise from their interaction with monoamine membrane transporters, leading to increased levels of catecholamines in the synaptic cleft and consequent sympathetic overstimulation.[2] However, the type of interaction and affinity toward the different transporters vary among derivatives.[8] In fact, methylone acts as a nonselective substrate for monoamine transporters, like MDMA and methamphetamine, although with inferior potency,[9] whereas MDPV functions as a pure transporter blocker, like cocaine, strongly inhibiting the uptake of dopamine (DA) and norepinephrine (NE), but with minimal effects on serotonin (5-HT) transporter.[10] Subjective effects differ among derivatives. Methylone induces MDMA-like stimulating effects, such as euphoria, increased energy and openness, mood lift and empathogenic feelings.[11] On the other hand, due to its affinity toward DA and NE transporters (DAT

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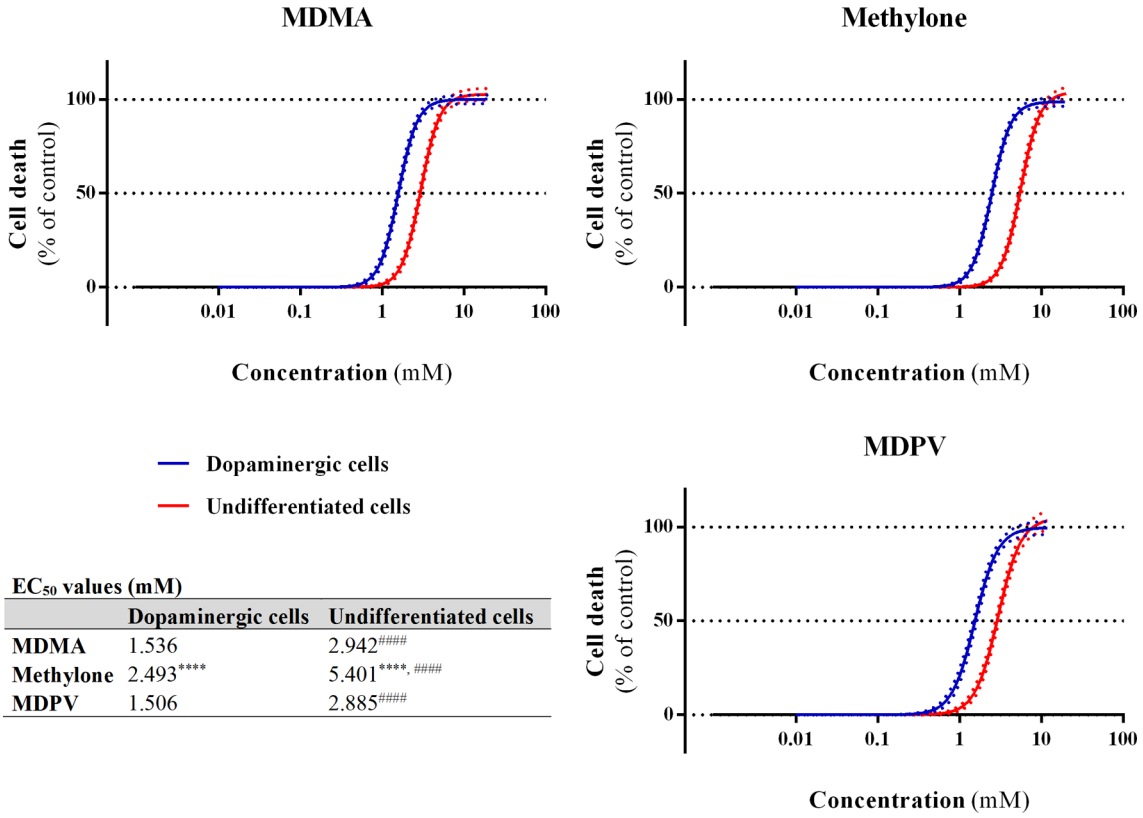

EC_{50} values (mM)

	Dopaminergic cells	Undifferentiated cells
MDMA	1.536	2.942$^{\#\#\#\#}$
Methylone	2.493****	5.401$^{****, \#\#\#\#}$
MDPV	1.506	2.885$^{\#\#\#\#}$

Figure 1. Nonlinear regression models for the cell death induced by MDMA, methylone and MDPV in undifferentiated and dopaminergic SH-SY5Y cells, as evaluated by the MTT reduction assay after 24 h exposure. The mean effects were fitted to the logit function. Dotted lines represent the 95% confidence band of each fit. Results were obtained from four independent experiments, performed in triplicate. Table: estimated EC_{50} values for each compound in both cell models. $^{****}p < 0.0001$ vs MDMA. $^{\#\#\#\#}p < 0.0001$ vs dopaminergic cells.

and NET), MDPV induces more cocaine-like subjective effects, including increased alertness and sexual stimulation, but limited euphoria and only mild, if any, empathogenic effects.[12,13] Higher doses and long-term abuse of these substances lead to unwanted psychological effects, including anxiety and panic attacks, hallucinations and psychosis.[14]

Neurotoxic effects of non-keto amphetamines have been thoroughly reported, and the main mechanisms by which these substances induce neuronal damage include neuroinflammation, excitotoxicity, oxidative stress and mitochondrial damage.[15−17] In vitro studies regarding the ability of cathinone derivatives to induce oxidative stress and mitochondrial dysfunction have recently been published, providing some light on the mechanisms possibly involved in β-keto amphetamines cytotoxicity in hepatic[18−21] and brain cells.[21−25] However, studies at a cellular level on the mechanisms of synthetic cathinones-induced neurotoxicity are still scarce and require clarification. Therefore, in the present work, we assessed the neurotoxic potential of methylone and MDPV in cultured human dopaminergic cells and investigated the mechanisms related to oxidative stress, mitochondrial dysfunction, and type of cell death.

RESULTS AND DISCUSSION

Dopaminergic Phenotype Renders SH-SY5Y Cells More Susceptible to the Cytotoxicity of β-Keto Amphetamines. Though the undifferentiated SH-SY5Y cell line expresses several dopaminergic markers, differentiated SH-SY5Y cells are more similar to primary neurons, with a mature neuron-like phenotype.[26] The desired phenotype determines the differentiation method. Generally, amphetamines and β-keto amphetamines present a strong affinity toward DAT, though with different potencies of binding and transporter-mediated release and/or uptake of monoamines. For instance, although the DAT binding affinity of methylone was shown to be 2.4-times higher than that of MDMA,[27] the potency of actual DAT-mediated release induced by this β-keto amphetamine appears to be much lower than its non-keto analogue.[9,27] On the other hand, MDPV, which presents substantial membrane permeability and is actively transported through the blood-brain barrier (BBB), was shown to have a binding affinity to DAT 650-times higher than MDMA[27] and strongly inhibits DAT-mediated DA reuptake, with an effectiveness 51-times over cocaine[10] and 548-times over MDMA.[27] Considering this strong interaction of β-keto amphetamines with DAT, RA-TPA exposure was the selected method for the present study, which prompts cell differentiation into a more dopaminergic phenotype.[28] In order to evaluate the relevance of differentiation to the neurotoxicity elicited by the β-keto amphetamines, the neurotoxic potential of MDMA, methylone and MDPV was measured through the MTT reduction assay in both undifferentiated and RA-TPA differentiated cells after a 24 h exposure. Figure 1 presents the concentration−response curves for each drug in the two in vitro models, as well as the estimated EC_{50} for each compound. With curve fits significantly shifted to the right, undifferentiated SH-SY5Y cells proved to be more resistant to drug-induced cell death ($p < 0.0001$). Previous work by our group[29] and others[30,31] provided evidence that RA-TPA differentiation elicits the rise in the expression of dopaminergic markers, including DAT and

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tyrosine hydroxylase, the rate-limiting enzyme of catecholamine biosynthesis. In order to determine if the increase in DAT expression could explain the higher susceptibility of differentiated cells to MDMA, methylone and MDPV-induced neurotoxicity, an additional study was conducted in the presence of GBR 12909, a selective DAT inhibitor. However, no protective effect of the DAT inhibitor was observed, even for the DAT substrates MDMA and methylone (Figure S1). These results suggest that, even though the neurotoxic profile of β-keto amphetamines may vary with the phenotype expressed by the neurons, their toxicity is independent of DAT-mediated uptake, possibly relying on the diffusion across the membrane.[27] In line with these results, our group also demonstrated that the cytotoxicity induced by MDMA for 48 h, in the same differentiated SH-SY5Y cells, is not significantly affected by the pre- and coexposure to the DAT inhibitor GBR 12909.[32] Work from den Hollander et al.[23] also showed no protective effect of this DAT inhibitor in methylone-induced LDH leakage in SH-SY5Y cells, though it significantly and completely reversed the cytotoxicity elicited by its membrane insoluble breakdown product 3,4-methylenedioxy-*N*-methylbenzamide, whose uptake occurs exclusively through DAT, corroborating the DAT-independent methylone-induced neurotoxicity. Whether the higher sensitivity of dopaminergic SH-SY5Y cells to the cytotoxicity elicited by both cathinones and MDMA may be related to different intrinsic metabolic capacities or redox cycle with DA is not known and requires future investigation.

Since all substances under study are strong dopaminergic drugs[9,10] and RA-TPA differentiation renders cells more sensitive to the cytotoxicity elicited by both cathinones and MDMA, all further toxicological evaluations were carried out in this cell model.

β-Keto Amphetamines Induce Neuronal Cell Death in a Concentration-Dependent Manner. As presented in Figure 1, exposure to the β-keto amphetamines resulted in concentration-dependent neuronal death, indicated by the decrease in MTT reduction, a marker of mitochondrial dysfunction. When comparing EC_{50} values estimated for this assay, MDPV and MDMA showed comparable potency in this in vitro model (EC_{50} of 1.506 and 1.536 mM, respectively), while methylone was significantly less potent than its non-keto analogue (EC_{50} of 2.493 mM, $p < 0.0001$ vs MDMA). This decrease in mitochondrial function was followed by a loss in membrane integrity, as determined through the LDH leakage assay (Figure 2). The LDH leakage assay proved to be less sensitive than the MTT reduction assay in determining cell viability, presenting significantly higher EC_{50} values than the latter (see embedded tables in Figures 1 and 2). In fact, mitochondrial dysfunction often precedes membrane damage, and the greater sensitivity of the MTT reduction assay over the LDH leakage measurement was already demonstrated in the past.[33] In accordance with our results, a recent study by Wojcieszak et al.[21] showed that MDPV did not trigger LDH leakage in undifferentiated SH-SY5Y cells at concentrations that already exhibited significant decrease in MTT reduction. Nevertheless, the order of drug potency determined by the LDH leakage assay was similar to that of MTT reduction assay: MDPV ≈ MDMA > methylone ($p < 0.0001$ vs MDPV and MDMA). Our group has recently showed comparable differences in potency of these three compounds in primary rat hepatocytes.[19] Structural differences among the tested drugs may underlie the difference in potency herein found. An overall

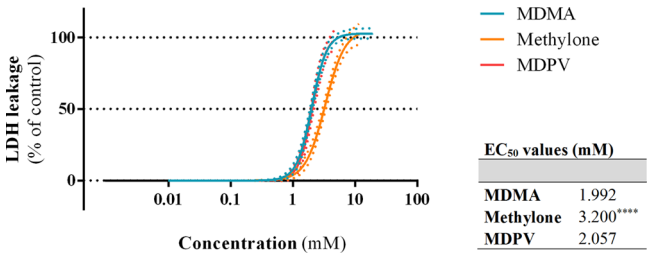

Figure 2. Nonlinear regression models for LDH leakage induced by MDMA, methylone and MDPV in dopaminergic SH-SY5Y cells after 24 h exposure. The mean effects were fitted to the logit function. Dotted lines represent the 95% confidence band of each fit. Results were obtained from four independent experiments, performed in triplicate. Table: estimated EC_{50} values for each compound in both cell models. ****$p < 0.0001$ vs MDMA.

increase in polarity and, consequently, a decline in lipophilicity and ability to cross phospholipidic bilayers is expectable from the presence of the β-keto group in the structure of synthetic cathinones.[27] Conversely, the pyrrolidine group present in derivatives such as MDPV greatly decreases their polarity,[12] counteracting the effect of the β-keto group.

Oxidative Stress as a Mechanism of β-Keto Amphetamines-Induced Neurotoxicity. The intracellular formation of ROS and RNS and levels of GSH and GSSG were measured in dopaminergic SH-SY5Y cells with the purpose of evaluating the influence of oxidative stress in the observed β-keto amphetamines-induced neurotoxicity. Contrarily to MDMA, which was unable to trigger the production of ROS and RNS at the conditions herein tested, methylone and MDPV induced a significant and concentration-dependent increase in reactive species formation after a 24 h exposure (Figure 3). This

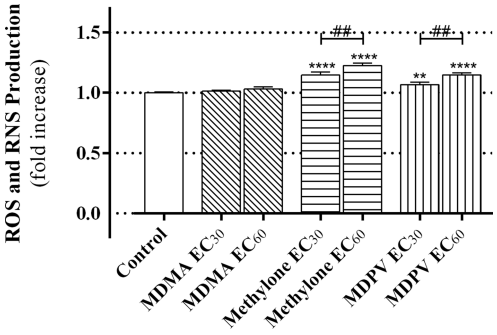

Figure 3. ROS and RNS production in dopaminergic SH-SY5Y cells exposed to EC_{30} and EC_{60} MDMA, methylone and MDPV for 24 h. Results were obtained from four independent experiments, performed in triplicate. **$p < 0.01$, ****$p < 0.0001$ vs control. ##$p < 0.01$ vs EC_{30}.

production was more evident for methylone, for which the EC_{60} induced a 1.23 ± 0.02-fold increase over control cells ($p < 0.0001$ vs control; $p < 0.01$ vs EC_{30} methylone). Our group recently showed that synthetic cathinones, contrarily to MDMA, present a great reducing potential,[19] which may justify our results in neuronal cells. We have hypothesized that a higher reducing potential may render the compounds more liable to enter redox cycling, thus promoting greater ROS generation. Nevertheless, further studies are required to ascertain this hypothesis in a cellular model. In accordance with our results, Rosas-Hernandez et al.[24] recently showed the ability of MDPV to trigger the formation of reactive species in

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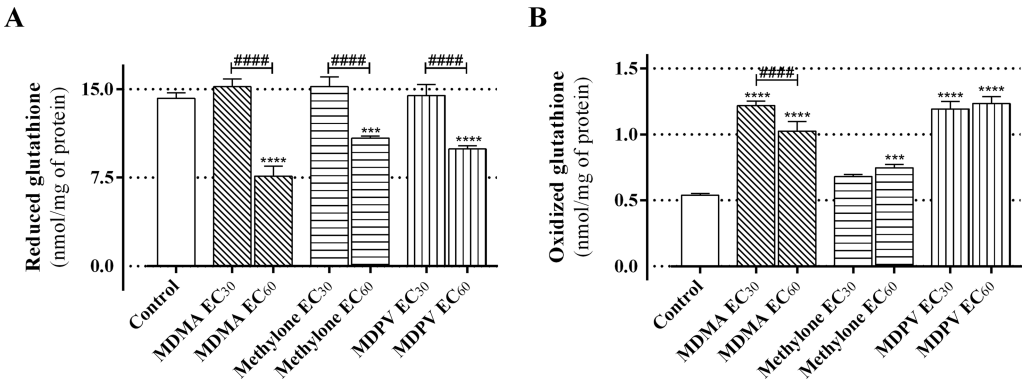

Figure 4. Intracellular levels of (A) GSH and (B) GSSG in dopaminergic SH-SY5Y cells exposed to EC_{30} and EC_{60} MDMA, methylone, and MDPV for 24 h. Results were obtained from three independent experiments, performed in triplicate. ***$p < 0.001$, ****$p < 0.0001$ vs control. $^{\#\#\#\#}p < 0.0001$ vs EC_{30}.

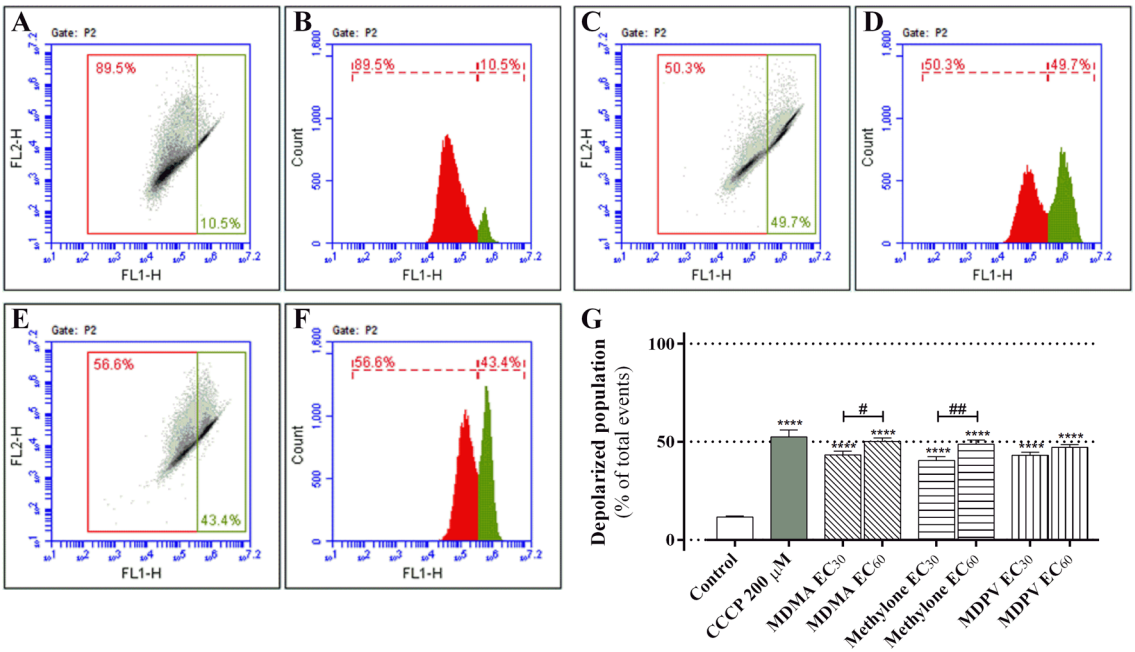

Figure 5. Representative cytometer plots displaying (A, C, and E) the distribution of events in FL2 vs FL1 and (B, D, and F) event counting and distribution in FL1 for (A and B) control cells, (C and D) 200 μM CCCP, and (E and F) EC_{30} MDPV. (G) Percentage of depolarized events in dopaminergic SH-SY5Y cells exposed to EC_{30} and EC_{60} MDMA, methylone and MDPV for 24 h. 200 μM CCCP was used as a positive control. Results were obtained from five independent experiments, performed in triplicate. ****$p < 0.0001$ vs control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs EC_{30}.

undifferentiated SH-SY5Y cells. When comparing MDPV and MDMA with regards to ROS production, Rosas-Hernandez et al.[25] also determined that this β-keto amphetamine is more potent than the non-keto amphetamine in an in vitro BBB model. Oxidative stress has been extensively implicated in the neurotoxicity of amphetamines, and it is characterized by a rise in ROS and RNS formation through several possible mechanisms, namely, oxidative deamination of catecholamines by monoamine oxidase, mitochondrial dysfunction, metabolism of the methylenedioxy ring, excitotoxicity, microglial activation, depletion of antioxidant systems and hyperthermia.[16,34,35] However, although evidence show that methamphetamine increases the formation of ROS in SH-SY5Y cells at millimolar levels,[36−38] to the extent of our knowledge, neuronal MDMA-induced ROS production was never shown in this in vitro model. In fact, after 24 h exposure to MDMA ($\leq$400 μM), our group found no signs of neuronal oxidative stress,[32,39,40] and only after 48 h was it possible to observe a significant decrease in GSH intracellular levels of MDMA-treated SH-SY5Y cells.[40] Evidences show that methylone and MDPV undergo metabolic pathways analogous to MDMA, including the formation of catechols.[41,42] The further oxidation of cathecols into highly reactive *ortho*-quinones that enter redox cycling is a well-known pathway for MDMA-induced ROS generation,[34] and may be also anticipated for synthetic cathinones containing a methylenedioxy ring, like methylone and MDPV. Moreover, the conversion of unsequestered DA into reactive quinones and radicals has been shown to contribute to methamphetamine-induced neurotoxicity,[43] and similar oxidative effects may be expected from the DA accumulation in the presynaptic cleft triggered by methylone and MDPV.[9,10] Additionally, the rise in intracellular ROS and RNS formation as a consequence of the disruption of the mitochondrial respiratory chain is also acknowledged,[44] and recent studies support the ability of synthetic cathinones to impair mitochondrial respiratory function in vitro,[19,20,22,23] including the present work (as

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shown below). The increase in ROS and RNS formation was followed by a significant GSH depletion, the primary cellular antioxidant barrier, at the EC_{60} of both β-keto amphetamines (Figure 4A), with a mean decrease of 23.0 ± 2.8 and 29.1 ± 3.8% for methylone and MDPV, respectively ($p < 0.0001$ vs control). For MDMA, GSH depletion was more pronounced, with a decrease of 44.7 ± 7.3% over control ($p < 0.0001$). This decrease in GSH levels was accompanied by a significant increased GSSG formation (Figure 4B), the oxidized form of GSH. The observed decline in GSH and increase in GSSG formation are likely consequences of the triggering of ROS and RNS generation. Interestingly, we observed GSH depletion for all three compounds at the EC_{60}, though no significant ROS and RNS production was found for EC_{60} of MDMA. In addition to its function as a direct superoxide anion and hydroxyl radical scavenger, GSH is also capable of conjugating with *ortho*-quinones to form glutathionyl adducts, which is a well described mechanism for amphetamines, including MDMA,[34] and may also justify the observed GSH depletion. Importantly, the formation of GSH conjugates was recently demonstrated *in vitro* for methylenedioxy β-keto amphetamines including MDPV and methylone.[45]

β-Keto Amphetamines Impair Mitochondrial Function in Dopaminergic Neuronal Cells. Mitochondrial oxidative phosphorylation is the main pathway for ATP synthesis, and is primarily stimulated by the mitochondrial uptake of free calcium, which in turn is driven by the Δψm.[44] Therefore, a disruption of Δψm may lead to mitochondrial dysfunction and disturb the intracellular energetic status. As depicted in Figure 5, methylone and MDPV, like MDMA, were shown to induce substantial mitochondrial depolarization. In fact, the Δψm dissipation was similar for all three substances, with significant increases already observed at the EC_{30}, from 11.6 ± 0.6% of depolarized events in control cells to 43.2 ± 2.1, 40.5 ± 1.9 and 43.1 ± 1.6% in cells treated with MDMA, methylone and MDPV, respectively ($p < 0.0001$). The Δψm dissipation has been associated with the neurotoxicity of amphetamine and methamphetamine,[46−48] hepatotoxicity of MDMA and methylone,[18] and khat-induced cell death of primary normal human oral cells.[49] This effect on Δψm was followed by a significant and concentration-dependent ATP depletion (Figure 6), with mean reductions of 66.1 ± 2.1, 27.0 ± 1.6 and 49.8 ± 3.3% as compared to control for EC_{60} MDMA, methylone and MDPV, respectively ($p < 0.0001$ vs control; $p < 0.01$ vs EC_{30}). Importantly, the decrease in GSH levels occurred at the highest concentration tested, whereas ATP depletion and/or mitochondrial membrane depolarization were already significant at EC_{30}, suggesting that mitochondrial dysfunction may be involved in the observed oxidative stress, as it was thoroughly reviewed for amphetamines-induced neurotoxicity.[15]

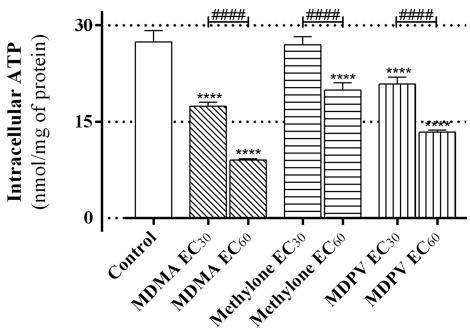

Figure 6. Intracellular levels of ATP in dopaminergic SH-SY5Y cells exposed to EC_{30} and EC_{60} MDMA, methylone, and MDPV for 24 h. Results were obtained from three independent experiments, performed in triplicate. ****$p < 0.0001$ vs control. ####$p < 0.0001$ vs EC_{30}.

β-Keto Amphetamines Induce Apoptotic Neuronal Cell Death in a Concentration-Dependent Manner. Subsequently to oxidative damage, mitochondria may undergo profound changes, with opening of the mitochondrial permeability transition pore (PTP), which leads to membrane depolarization (Δψm dissipation), that in turn signals the translocation of the pro-apoptotic factor Bax to the mitochondria, causing cytochrome *c* release through the PTP and initiating an apoptotic signaling cascade that includes the activation of caspase 9 and the effector caspase 3.[44,50,51] Accordingly, a significant rise in caspases 3 and 9 activity was observed for both β-keto amphetamines, in a concentration-dependent manner (Figures 7A and C). For the EC_{60}, methylone presented an increase of 336.9 ± 20.1 and 170.8 ± 14.0% of caspase 3 and 9 activity over control ($p < 0.0001$), respectively, while for MDPV this increment was slightly higher: 346.2 ± 29.9 and 184.1 ± 10.5% of activity over control ($p < 0.0001$). For both β-keto compounds, but not MDMA, it was also observed a significant activation of caspase 8 (Figure 7B), with similar values for EC_{60} methylone and MDPV: 153.3 ± 14.3 and 156.6 ± 13.5% of activity over control ($p < 0.001$). The activation of caspase 8 independently portrays the mitochondria-independent pathway of apoptosis, as a consequence of the binding of the drugs to cell surface death receptors, leading to further activation of the effector caspases, and/or indirect activation of the mitochondria-dependent pathway.[52] The activation of both intrinsic and extrinsic apoptotic mechanism was also reported for methylone and MDPV in primary rat hepatocytes[19] and for MDMA in neuronal in vivo and in vitro models.[53,54]

Apoptosis is characterized by a series of typical morphological features such as cell shrinkage, chromatin condensation, nuclear fragmentation and plasma membrane blebbing.[55] Experimental evidence has revealed nuclear morphological changes associated with neuronal cell death induced by amphetamine derivatives, following mitochondria swelling, release of pro-apoptotic factors, activation of caspases and DNA fragmentation.[56−60] Here we also demonstrated, through Hoechst 33342/PI double staining, the triggering of apoptosis in dopaminergic SH-SY5Y cells exposed to the β-keto amphetamines, with evident changes in the morphology of chromatin (Figure 8). In nontreated cells, large blue nuclei appear with regular contours, while exposure to the drugs leads to chromatin condensation characterized by a reduction of the nuclei size (e.g., EC_{60} MDPV compared to control cells), and presence of pyknotic nuclei, distinctive of early apoptosis (without PI label, green arrows) and late apoptosis (with PI label, yellow arrows). For the tested conditions, cells appear to be in advanced stages of apoptosis, with a general prevalence of late apoptotic events. Necrotic cells (large PI-labeled nuclei, red arrows) were scarce. Nuclear morphology data further supports the neuronal β-keto amphetamines-induced activation of apoptosis, with an evident increase of apoptotic events in a concentration dependent manner.

Importantly, the concentrations herein studied are considerably higher than the micromolar levels found in blood samples from β-keto amphetamines-related intoxications,[61,62] as well as the brain distribution levels determined in postmortem samples.[63] Nonetheless, the concentrations are in the range of

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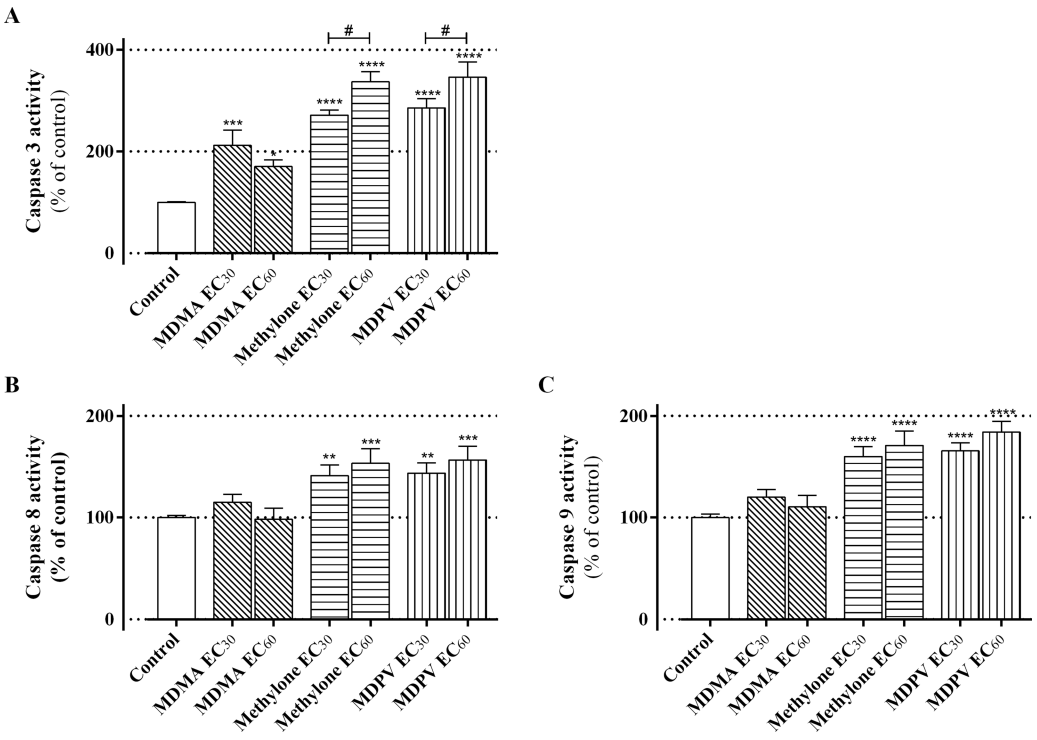

Figure 7. Caspase (A) 3, (B) 8, and (C) 9 activity in dopaminergic SH-SY5Y cells exposed to EC_{30} and EC_{60} MDMA, methylone and MDPV for 24 h. Results were obtained from four independent experiments, performed in duplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs control. $^{\#}p < 0.05$ vs EC_{30}.

those used in other in vitro studies of this nature[18,23] and the obtained data should be considered as an insight into the cellular mechanisms that may be involved in the in vivo effects of these NPS. It is also important to stress that the metabolic pathways of β-keto amphetamines might lead to the formation of highly neurotoxic metabolites, which, individually or in mixture, may induce deleterious effects significantly more pronounced than the parent compound, through similar mechanisms but at substantially lower concentrations, as it was recently demonstrated by our group for MDMA and its metabolites.[29] Additionally, further studies on the neurotoxicity of β-keto amphetamines at in vivo relevant concentrations are currently underway.

CONCLUSIONS

The continuous growth in the use of cathinone designer drugs as alternative drugs of abuse poses significant public health challenges. The current work provided evidence of the in vitro neurotoxic potential of two commonly abused β-keto amphetamines. A common pathway of neurotoxicity was found for methylone and MDPV, which involved oxidative stress, characterized by ROS and RNS production and GSH depletion, and mitochondrial dysfunction, with $\Delta\psi m$ dissipation and ATP depletion, ultimately leading to cell death by apoptosis, with activation of both mitochondria-dependent and -independent pathways. Better knowledge of these mechanisms may help to outline therapeutic approaches to avoid or attenuate the known adverse outcome of synthetic cathinones abuse.

METHODS

Chemicals. Hydrochloride salts of methylone and MDPV were purchased online from the Sensearomatic Web site (currently unavailable) during March 2013. MDMA was extracted, purified, and converted to the respective hydrochloride salt at UCIBIO-REQUIMTE, Laboratory of Toxicology, Faculty of Pharmacy, Porto, Portugal, from high purity MDMA tablets provided by the Portuguese Criminal Police Department. The salts were fully characterized by mass spectrometry, NMR, and elemental analysis, and purity was greater than 98%. Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, retinoic acid (RA), 12-*o*-tetradecanoylphorbol 13-acetate (TPA), thiazolyl blue tetrazolium bromide (MTT), sodium pyruvate, β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (β-NADH), L-glutathione reduced (GSH) and L-glutathione oxidized disodium salt (GSSG), β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (β-NADPH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), adenosine triphosphate (ATP), luciferase from *Photinus pyralis* (firefly) and D-luciferin sodium salt, bisbenzimide H 33342 trihydrochloride (Hoechst 33342), propidium iodide (PI), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Heat-inactivated fetal bovine serum (FBS), antibiotic mixture of penicillin/streptomycin (10 000 U/mL/10 000 μg/mL), MEM Non-Essential Amino Acids (NEAA) 100×, and Hank's balanced salt solution (HBSS) were obtained from GIBCO Invitrogen (Barcelona, Spain). 1-(2-[Bis(4-fluorophenyl)-methoxy]ethyl)-4-(3-phenylpropyl)piperazine (GBR 12909) hydrochloride was provided by Cayman Chemical (Ann Arbor, MI). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazoylcarbocyanine iodide (JC-1 dye) and 2-[2-(3-chlorophenyl)hydrazinylyidene]-propanedinitrile (CCCP) were supplied by Thermo Fisher Scientific (Waltham, MA) and Abcam (Cambridge, U.K.), respectively. All other chemicals of analytical grade were purchased from Merck (Darmstadt, Germany).

Cell Culture Conditions. The SH-SY5Y cell line is a human neuroblastoma cell line that retain neuronlike properties, thus being widely used as neuronal model.[64] SH-SY5Y cells were supplied by ATCC (American Tissue Culture Collection, Manassas, VA), and routinely maintained in complete DMEM medium, supplemented with 10% FBS, 1% MEM NEAA, and 1% penicillin/streptomycin, at 37 °C in a humidified atmosphere of 5% CO_2. Subcultures were performed weekly by trypsinization, and the assays were conducted over 8

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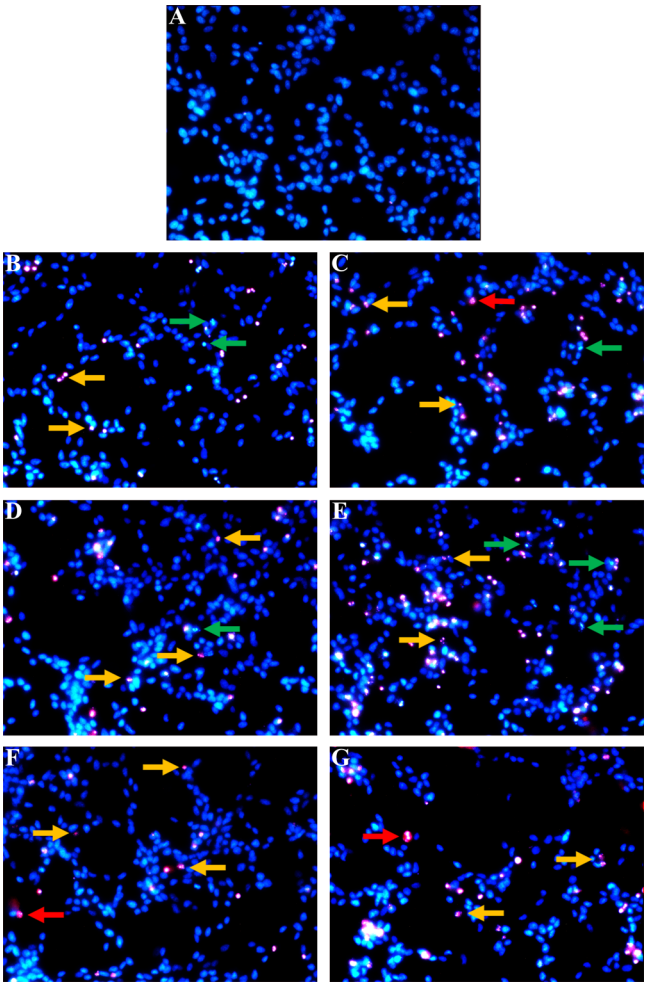

Figure 8. Representative fluorescence microscopy images of Hoechst 33342/PI double staining of (A) control dopaminergic SH-SY5Y cells, and cells exposed to EC_{30} and EC_{60} (B and C) MDMA, (D and E) methylone, and (F and G) MDPV for 24 h. Green arrows indicate early apoptotic cells (blue condensed nuclei), yellow arrows indicate late apoptotic cells (red condensed nuclei), and red arrows indicate necrotic cells (large red nuclei). Original magnification ×200.

passages (passages 32–39) to avoid phenotypic changes. Upon plating, cells were cultured at a density of 2.5×10^4 cells/cm^2 and differentiated into a dopaminergic phenotype prior to the incubation with compounds. SH-SY5Y cell differentiation was induced with 10 nM RA for 72 h, followed by 10 nM RA and 80 nM TPA for 72 h more. For viability assays conducted in undifferentiated cultures, cells were plated at 2.5×10^4 cells/cm^2 without differentiation factors, cell media was renewed after 72 h, and the drug treatments took place 72 h later.

Experimental Procedures. All drug treatments were performed in complete medium to avoid apoptotic cell death by serum deprivation.[65] In order to obtain a complete cytotoxicity concentration–response curve, undifferentiated and RA-TPA differentiated SH-SY5Y cells were exposed for 24 h to MDMA, methylone or MDPV at a wide concentration range, from 0.01 to 12 or 20 mM, and cell viability was indirectly evaluated through the MTT reduction assay, which provides an assessment of mitochondrial function. For RA-TPA differentiated cells, the integrity of the plasma membrane was also assessed by measuring the cellular leakage of the cytosolic enzyme lactate dehydrogenase (LDH) at the same concentration range. Further experiments were conducted only in RA-TPA differentiated cells, including ROS and RNS production, determination of GSH and GSSG levels, measurement of the mitochondrial membrane potential ($\Delta\psi$m) and ATP intracellular levels, determination of caspases 3, 8, and 9 activities, and Hoechst 33342/PI double staining for fluorescent microscopy. Cells were exposed to the drugs for 24 h, at the EC_{30} and EC_{60} estimated from the concentration–response curves obtained from the MTT reduction assay for this cell model. The determined concentrations for MDMA, methylone and MDPV were, in that order, as follows: EC_{30} of 1.253, 1.962, and 1.165 mM; EC_{60} of 1.693, 2.797, and 1.703 mM. In experiments where the effect of the selective DAT inhibitor GBR 12909 was tested, the cells were pretreated for 30 min with 1 μM GBR 12909 and then coincubated for 24 h with the drugs at the EC_{30} and EC_{60}.

Cell Viability Assays. Cell viability was assessed using the MTT reduction assay, which measures the mitochondrial function, as previously described[29] with minor modifications. Briefly, after 24 h of exposure, cells were incubated with a 0.5 mg/mL MTT solution for 30 min, after which the formazan crystals formed through mitochondrial succinate dehydrogenase were dissolved in DMSO and quantified at 550 nm in a 96-well plate reader (PowerWaveX; Bio-Tek, Winooski, VT). The LDH leakage was also assessed in RA-TPA differentiated cells, as described before.[20] The half maximal effective concentration (EC_{50}) of each compound was estimated for both data sets.

ROS and RNS Generation. The intracellular ROS and RNS generation was monitored by means of the DCFH-DA probe, as previously described.[20] Data was obtained from four independent experiments, run in triplicate, and normalized to cells with no treatment.

Intracellular GSH and GSSG Levels. Total glutathione (tGSH) and GSSG levels were determined by the DTNB–GSSG reductase recycling assay, as described before.[66] GSH levels were calculated by subtracting GSSG content from tGSH values as follows: GSH = tGSH − (2 × GSSG). Data were obtained from three independent experiments, run in triplicate, and normalized to total protein.

Mitochondrial Membrane Potential. The $\Delta\psi$m reflects the functional status of mitochondria within the cells. Changes in $\Delta\psi$m were evaluated through the uptake of the JC-1 dye into the mitochondria. JC-1 is a lipophilic cationic dye that selectively enters the mitochondria proportionately to the membrane potential. Color emission shifts reversibly from red to green upon membrane depolarization. Briefly, cells were trypsinized, centrifuged at 400*g* for 3 min and pellets were resuspended in a 5 μg/mL JC-1 solution and incubated at 37 °C for 20 min. Cells were then rinsed and resuspended in PBS for further analysis using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). Both polarized (red fluorescence) and depolarized cells (green fluorescence) were detected on the FL2 (585 nm) and FL1 (533 nm) channels. A minimum of 20 000 events per sample was acquired for analysis. A 200 μM solution of CCCP, a potent mitochondrial oxidative phosphorylation uncoupler, was used as positive control of depolarization. Results were obtained from five independent experiments run in triplicate.

Intracellular ATP Levels. Intracellular contents of ATP were measured as previously described,[39] through a bioluminescence assay based on the emission of light from the reaction of ATP and luciferin, catalyzed by the enzyme luciferase. Data were obtained from three independent experiments, run in triplicate, and normalized to total protein.

Caspases 3, 8, and 9 Activities. The activity of caspases 3, 8, and 9 was determined in the cytoplasmatic fractions, through a colorimetric assay using substrates for each caspase, as previously described.[20] Results were obtained from four independent experiments, run in duplicate, and normalized to total protein contents on the cell lysate.

Nuclear Morphological Modifications. The activation of nuclear morphological alterations by MDMA, methylone and MDPV in SH-SY5Y cells was determined using Hoechst 33342, a cell-permeant nuclear counterstain emitting blue fluorescence when bound to DNA, and PI, a membrane impermeant nuclear dye that emits red fluorescence in dead cells, as previously described.[20]

Statistical Analysis. Curves of normalized mortality values as a function of concentration, obtained through the MTT reduction and the LDH leakage assays, were constructed and analyzed as previously described,[19] with a modified logit function applied as follows: Y =

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$\theta_{max}/(1 + \exp(-\theta_1 - \theta_2 \log(x)))$, where θ_{max} is the maximal observed effect, x is the concentration of the test drug, θ_1 is the parameter for the location, and θ_2 is the slope parameter. Statistical uncertainties are expressed as 95% confidence intervals. For other assessments, results are presented as mean ± standard error of the mean (SEM). Normality of the data distribution was assessed by the D'Agostino & Pearson omnibus normality test. Multiple comparisons within each compound (concentration as a variable) or between each synthetic cathinone and MDMA were performed through one-way ANOVA analysis, followed by Fisher's LSD post hoc test. Nonlinear curve fitting and all statistical calculations were performed using GraphPad Prism 6 (version 6.01) for Windows. *P*-values lower than 0.05 were considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.6b00421.

Cytotoxicity induced by MDMA, methylone, and MDPV in differentiated SH-SY5Y cells in the presence or absence of DAT inhibitor GBR 12909 (PDF)

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Author Contributions

All authors contributed extensively to the discussion of the experimental data generated. M.J.V. carried out the experiments, analyzed data, and wrote the manuscript. E.F. extracted, purified, and converted MDMA pills to the respective hydrochloride salt. M.L.B., F.C., and P.G.P. gave conceptual advice and revised the manuscript. M.C. designed the experiments, supervised the project, and revised the manuscript.

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Notes

The authors declare no competing financial interest.

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Manuscript IV. Methylone and MDPV activate autophagy in human dopaminergic SH-SY5Y cells: a new insight into the context of β-keto amphetamines-related neurotoxicity

Submitted manuscript

Methylone and MDPV activate autophagy in human dopaminergic SH-SY5Y cells: a new insight into the context of β-keto amphetamines-related neurotoxicity

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Abstract

Autophagy has an essential role in neuronal homeostasis and its dysregulation has been recently linked to neurotoxic effects of a growing list of psychoactive drugs, including amphetamines. However, the role of autophagy in β-keto amphetamine (β-KA) designer drugs-induced neurotoxicity has hitherto not been investigated. In the present study, we show that two commonly abused cathinone derivatives, namely 3,4-methylenedioxymethcathinone (methylone) and 3,4-methylenedioxypyrovalerone (MDPV), elicited morphological changes consistent with autophagy and neurodegeneration, including formation of autophagic vacuoles and neurite retraction. Methylone and MDPV prompted the formation of acidic vesicular organelles (AVOs) and lead to increased expression of the autophagy-associated protein LC3-II in a concentration- and time-dependent manner. Electron microscopy confirmed the presence of autophagosomes with typical double-membranes and autolysosomes in SH-SY5Y cells exposed to both β-KA. The autophagic flux was further confirmed using bafilomycin A1, a known inhibitor of the late phase of autophagy. Moreover, we showed that autophagy markers were activated before the triggering of cell death and caspase-3 activation, suggesting that β-keto amphetamines-induced autophagy precedes apoptotic cell death. To address the role of oxidative stress in autophagy induction, we also investigated the effects of antioxidant treatment with *N*-acetyl-L-cysteine (NAC) on autophagy and apoptotic markers altered by these drugs. NAC significantly attenuated methylone- and MDPV-induced cell death, by completely inhibiting the generation of ROS and RNS, and hampering both apoptotic and autophagic activity, suggesting that oxidative stress plays an important role in mediating autophagy and apoptosis elicited by these drugs.

Introduction

β-Keto amphetamines (β-KA), also known as synthetic cathinones, emerged recently in the recreational setting as a new class of psychoactive substances with structural and pharmacological properties resembling those of well-known amphetamines (Valente et al. 2014; Zaitsu et al. 2011). With over a hundred β-KA derivatives detected so far (EMCDDA-Europol 2016), 3,4-methylenedioxymethcathinone (methylone) and 3,4-methylenedioxypyrovalerone (MDPV) are among the most widely consumed ones. Like amphetamines or cocaine, methylone and MDPV inhibit the uptake of biogenic amines at the central nervous system, acting respectively as substrate or inhibitor of dopamine, noradrenaline and serotonin transporters, and leading to the accumulation of these catecholamines in the synaptic cleft and subsequent overstimulation of the sympathetic system (Baumann et al. 2012; Baumann et al. 2013; Prosser and Nelson 2012).

Apart from their effects on the monoaminergic systems, little is known about the potential detrimental effects of β-KA to the brain. However, in view of the obvious similarities to long-studied neurotoxic drugs of abuse, the increasing abuse of β-KA raises concerns about potential neurotoxic effects. The first studies addressing the *in vitro* neurotoxicity of β-KA emerged in the past few years (den Hollander et al. 2014; den Hollander et al. 2015; Rosas-Hernandez et al. 2016a; Rosas-Hernandez et al. 2016b; Valente et al. 2017; Wojcieszak et al. 2016) indicating that neuronal death occurs through apoptosis and necrosis. Notwithstanding, several lines of evidence support that another form of programmed cell death, namely autophagic cell death, may play an important role in human neurodegenerative diseases (Jaeger and Wyss-Coray 2009). More recently, a growing number of psychoactive substances were also shown to induce autophagy, including cocaine (Cao et al. 2016; Guo et al. 2015; Periyasamy et al. 2016), MDMA (Li et al. 2016; Li et al. 2014; Mercer et al. 2017), methamphetamine (Chandramani Shivalingappa et al. 2012; Kanthasamy et al. 2006; Larsen et al. 2002; Li et al. 2012), cannabinoids (Salazar et al. 2009), morphine (Zhao et al. 2010), and ethanol (Chen et al. 2012; von Haefen et al. 2011).

Autophagy upregulation was shown to be involved in the neurotoxicity triggered by amphetamine derivatives, though the role of autophagic activation in the observed neurodegeneration remains a controversial matter. For instance, the autophagic inhibitor 3-methyladenine significantly attenuated MDMA-induced autophagic activation, while ameliorating MDMA-triggered neurite damage and neuronal death in both primary cultured cortical and serotonergic neurons (Li et al. 2016; Li et al. 2014). In contrast, Mercer et al. (2017) found that the potentiation of autophagy by rilmenidine, a known

autophagy activator, also had beneficial effects against MDMA-elicited neurodegeneration in serotonergic neurons, supporting a neuroprotective role for autophagy. Methamphetamine was shown to induce autophagy in dopaminergic cells by modulating regulatory factors common to apoptotic and autophagic pathways (Nopparat et al. 2010), and this autophagic upregulation was further determined to precede apoptotic activation (Kanthasamy et al. 2006). Moreover, the antioxidant agents *N*-acetyl-L-cysteine, L-ascorbate, taurine and melatonin exhibited significant protective effects against the neurotoxic effects of methamphetamine by downregulating both cell death pathways (Chandramani Shivalingappa et al. 2012; Huang et al. 2017; Kongsuphol et al. 2009; Li et al. 2012; Nopparat et al. 2010).

Regarding the β-KA, recent studies showed that they promote the production of reactive oxygen species, which have been proposed to act as upstream modulators of autophagy induction (Li et al. 2015). However, whether autophagy is activated by these drugs or its role in β-KA-induced neurotoxicity remains unknown. For this reason, the present study aimed at evaluating the occurrence and contribution of autophagy to neurotoxicity in dopaminergic SH-SY5Y cells challenged with methylone and MDPV. Our results provide evidence of autophagy activation by methylone and MDPV in dopaminergic SH-SY5Y cells. Moreover, NAC treatment ameliorated both methylone- and MDPV-induced autophagy and decreased the associated toxicity, suggesting that oxidative stress participates in the control of autophagy in response to β-KA in SH-SY5Y cells.

Materials and Methods

Chemicals

Methylone and MDPV hydrochloride salts were purchased online (website currently unavailable), during March 2013. Chemical purity and identity of these compounds were verified by mass spectrometry, NMR and elemental analysis. All analytical data were consistent with the assigned structures with over 98% purity for both cathinone derivatives. Dulbecco's Modified Eagle's Medium (DMEM) - high glucose, thiazolyl blue tetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA), acridine orange (AO), *N*-acetyl-L-cysteine (NAC), monoclonal anti-α-tubulin antibody, retinoic acid (RA), 12-O-tetradecanoylphorbol 13-acetate (TPA), bafilomycin A1 (Baf A1) from *Streptomyces griseus* and bovine serum albumin (BSA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Heat-inactivated fetal bovine serum (FBS), antibiotic

mixture of penicillin/streptomycin (10,000 U/mL / 10,000 µg/mL), MEM Non-Essential Amino Acids 100 X, trypsin-EDTA and Hank's balanced salt solution (HBSS) were obtained from Thermofisher Scientific (Waltham, MA, USA). Rabbit polyclonal anti-LC3A/B antibody was supplied by Cell Signaling Technology® (Danvers, MA, USA). Amersham™ ECL™ Western Blotting reagent pack containing secondary anti-mouse and anti-rabbit HRP conjugate antibodies was purchased from GE Healthcare Life Sciences (Marlborough, MA, USA). All other chemicals of analytical grade were purchased from Merck (Darmstadt, Germany) or Sigma Aldrich (St. Louis, MO, USA).

Cell culture and differentiation

The epithelial SH-SY5Y human neuroblastoma derived cell line was supplied by ATCC® (American Tissue Culture Collection, Manassas, VA), and routinely maintained in complete DMEM medium, supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and MEM Non-Essential Amino Acids, at 37ºC, in a humidified atmosphere of 5 % CO_2. Cells were subcultured over 7 passages (passages 31 to 37), and cultured at a density of 25.000 cells/cm^2. Upon plating, cell differentiation into a dopaminergic phenotype was induced using 10 nM RA for 72 h, followed by 10 nM RA and 80 nM TPA for 72 h more.

Experimental design

Exposure to the test drugs took place after complete cell differentiation and was performed in complete medium to avoid the induction of autophagic cell death by serum starvation (Macleod et al. 2001; Xu et al. 2013). Complete cytotoxicity concentration-response curves for differentiated SH-SY5Y cells exposed for 24 h to methylone and MDPV were previously determined through the MTT reduction assay (Valente et al. 2017). For the present experiments, the β-KA drugs were tested at the effective concentrations (ECs) 10, 30 and 60, i.e., the concentrations that induce 10, 30 and 60% of viability loss, respectively, as estimated from the concentration-response curves. The determined ECs for methylone and MDPV were, in that order, as follows: EC_{10} of 1.342 and 0.773; EC_{30} of 1.962 and 1.165 mM; EC_{60} of 2.797 and 1.693 mM. Depending on the experiment, cells were exposed to the drugs for 2, 6 and/or 24 h. For the assessment of the combination of each drug with the autophagy inhibitor Baf A1, cells were co-treated for 24 h with EC_{60} methylone or MDPV and 40 nM Baf A1, whereas for the antioxidant NAC, cells were pretreated for 30 min with 5 mM NAC before adding the drugs at EC_{60} for 24 h more.

Phase contrast microscopy

To detect possible morphological modifications due to the exposure to methylone or MDPV, cells were rinsed with HBSS after incubation with the compounds, and observed under an inverted microscope Nikon Eclipse TS100F (Nikon, Amsterdam, Netherlands), at an original magnification of 200×.

Fluorescence microscopy with acridine orange staining

Following exposure to methylone or MDPV, cells were incubated with 0.5 µg/mL AO for 20 min, at 37 ºC, to detect AVOs formation. AO is a lysosomotropic dye that emits green/yellow/orange/red fluorescence in a pH-dependent manner. At neutral pH, AO is a hydrophobic green molecule, while within acidic organelles it becomes protonated and form aggregates that emit bright yellow to red fluorescence. Following incubation with AO, cells were rinsed three times with HBSS and observed under an inverted fluorescence microscope Nikon Eclipse Ti (Nikon, Amsterdam, Netherlands), at an original magnification of 200×.

Flow cytometry with acridine orange staining

In order to quantify the drug-induced formation of AVOs, cells exposed to the drugs were collected with trypsin-EDTA, rinsed with HBSS and incubated with 0.5 µg/mg AO for 20 min, at 37 ºC, with agitation. Cells were then rinsed three times with HBSS by centrifugation at 400*g* for 3 min, and resuspended in HBSS for further analysis in a BD Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA, USA), using two channel (FL3/FL1) detection set on a linear scale. Briefly, the nucleus and cytoplasm of acridine orange-stained cells emit green fluorescence (FL1, 533 nm), whereas AVOs emits red fluorescence (FL3, 675 nm). Thus, the increase in intensity of red fluorescence (AO^+ events), is proportional to the volume of AVOs. A minimum of 20,000 events per sample was acquired for analysis. Results are from four independent experiments, run in triplicate. Data were analyzed using BD Accuri™ software.

Transmission electron microscopy

To determine the nature of the observed vacuoles in phase contrast microscopy, cells exposed to methylone or MDPV were analyzed through transmission electron microscopy (TEM). For this purpose, about 5.0 x 10^6 cells were collected by

trypsinization, rinsed with HBSS, and centrifuged at 130*g* for 3 min. The pellets were then immersed in a solution of 2.5 % gluteraldehyde in 0.1 M cacodylate buffer, pH 7.2, for two hours. After rinsing with cacodylate buffer, tissue specimens were post-fixed in 2% osmium tetroxide and rinsed again prior to dehydration with graded ethanol and further embedding in Epon™. The samples were then stored for two days at 60 ºC to promote resin polymerization. Ultrathin (100 nm) sections, contrasted with uranylacetate and lead citrate, were prepared on cupper grids (300 Mesh) for TEM analysis at 80 kVolts in a JEOL USA JEM-1400 Transmission Electron Microscope (JEOL USA, Inc., Peabody, MA, USA).

LC3 Western Blotting analysis

Drug-induced LC3 turnover was detected through Western Blotting. For this purpose, after treatment, cells were rinsed, lysed in cold lysis buffer (20 mM Tris HCl; 150 mM NaCl; 0.3% Triton X-100; 5 mM EDTA; pH 7.5) containing protease inhibitor cocktail, for 30 min at 4 ºC, and collected with a Teflon cell scrapper. Samples were then vortexed, sonicated and centrifuged at 15 000*g* for10 min. The supernatants were collected into new vials and the total protein content was determined using a Bio-Rad DC™ Protein Assay kit. A total of 100 µg of protein of each sample was subjected to 15% acrylamide/bis-acrylamide SDS-PAGE gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes, and the immunodetection was performed using a rabbit polyclonal anti-LC3A/B antibody (1:250), and the secondary anti-rabbit HRP conjugate antibody (1:2000). α-Tubulin was detected as a protein loading control, using a primary monoclonal anti-α-tubulin antibody (1:500) and a secondary anti-mouse HRP conjugate antibody (1:2000). Immunoreactive bands were visualized using the chemiluminescent Clarity™ Western ECL substrate and analyzed in a Bio-Rad ChemiDoc™ XRS+ System with Image Lab™ Software (Hercules, CA, USA). Results are from at least three independent experiments.

Cell viability assays

The viability of cells exposed to the different individual compounds or to the combination of each one with NAC was determined indirectly through the MTT reduction, performed as previously described (Valente et al. 2017). Data was obtained from five independent experiments, run in triplicate, and normalized to non-treated (100 % MTT reduction) and 1% triton X-100-treated cells (0 % MTT reduction).

ROS and RNS production

The intracellular levels of ROS and RNS were determined using the DCFH-DA fluorescent probe, as previously described (Valente et al. 2016b), with minor modifications. Briefly, after differentiation, cells were incubated with a 100 µM DCFH-DA solution in complete medium. After 30 min, the supernatant was discarded and cells were exposed to the drugs. For ROS and RNS measurement in the presence of NAC, cells were simultaneously pretreated for 30 min with 100 µM DCFH-DA and 5 mM NAC, and then exposed for 24 h with NAC alone or in combination with the drugs. Results were obtained from five independent experiments, performed in triplicate, and normalized to non-treated cells.

Caspase-3 activity

The cytoplasmic caspase-3 activity was measured as a marker of apoptotic activity, using a colorimetric substrate for this effector caspase, as previously described (Valente et al. 2016b). Data are from five independent experiments, run in duplicate, and normalized to total protein concentration in cell lysates.

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). Normality of the data distribution was assessed by the D'Agostino & Pearson omnibus normality test. Multiple comparisons within each compound (concentration or time as a variable) or between treatments were performed through one-way ANOVA analysis, followed by Fisher´s LSD post hoc test. All statistical calculations were performed using GraphPad Prism 6 (version 6.01) for Windows. P-values lower than 0.05 were considered statistically significant.

Results

β-keto amphetamines trigger autophagy in dopaminergic SH-SY5Y cells in a time- and concentration-dependent manner

Hitherto, there were no reports of the autophagy induction by β-KA on neuronal cells. In this study, we employed differentiated SH-SY5Y cells as an *in vitro* model of human dopaminergic neurons to evaluate the possible involvement of autophagy in

methylone- and MDPV-induced neurotoxic effects. The autophagy activation was investigated at different time points (2, 6 and 24 h) after initial exposure of cell to methylone or MDPV and with different drug concentrations (EC_{10}, EC_{30} and EC_{60}, i.e., effective concentrations causing respectively 10%, 30% and 60% loss of cell viability after 24 h exposure).

We first examined the morphological changes in treated cells by phase contrast microscopy. As shown in Fig. 1, a marked increase in cytoplasmic vacuolization was already visible in methylone-treated cells after 2 h exposure (Fig. 1D), which was not seen in control cells (Fig. 1A), suggesting the occurrence of autophagy. Similar results were obtained in MDPV-treated cells (data not shown). Vacuole formation was accompanied by neurite retraction, being these alterations more pronounced with time of incubation (Figs 1D to 1F) and drug concentration (data not shown).

Figure 1

To clarify the nature of these vacuoles, we next studied the presence of AVOs by fluorescence microscopy and flow cytometry. The volume of cellular acidic compartment has been reported as a marker of autophagy that can be visualized after cell staining with acridine orange (AO) (Paglin et al. 2001). As shown in Fig. 2, control cells showed minimal acidic fluorescence (Fig. 2B). In contrast, cells exposed to EC_{60} MDPV for 6 h present bright yellow/orange/red fluorescence within vesicles, whereas the nucleus and cytoplasm exhibit green fluorescence (Fig. 2D). Interestingly, immunofluorescence images demonstrated that AVOs appear not only in the cell body of the neurons (red arrow), but also in neurites (yellow arrow).

Red fluorescent events were also quantified through flow cytometry. The results show that methylone and MDPV induced AVOs formation in SH-SY5Y cells in a time- and concentration-dependent manner (Fig.s 2E and 2F). When comparing effects of the same drug concentration (EC_{30}) but at different time points (Fig. 2E), both methylone and MDPV elicited a significant increase of AO^+ events already after 2 h of exposure, with 13.8 ± 2.4 and 10.8 ± 0.7 % AO^+ events, respectively ($p<0.001$ *vs.* control). Both drugs significantly increased the AVOs formation already at the lowest concentration tested (Fig. 2F), with over 20 % of AO^+ events for EC_{10} methylone or MDPV compared to the 3 % detected in control cells ($p<0.0001$).

Figure 2

To further determine the effect of β-KA on autophagy activity, we analyzed the expression of autophagy protein LC3 by western blotting analysis. Upon autophagy activation, the cytosolic form of the LC3, or LC3-I, is cleaved, conjugated with phosphatidylethanolamine to form LC3-II, and this form is inserted into autophagosome membranes (Tanida et al. 2008). Thus, the cytosolic turnover of LC3-I to LC3-II, here graphically represented as the LC3-II/LC3-I ratio, is a hallmark of autophagy and correlates with autophagosome formation. The expression of LC3 proteins, as well as the ratios of LC3-II/LC3-I, at different concentrations and exposure times are depicted in Fig. 3. The LC3-II band, virtually absent in control cells, is evident in drug-treated cells (Fig. 3A). We observed an increase in LC3-II expression in cells exposed to the β-KA in a time- and concentration-dependent manner, being this effect more pronounced for methylone. The results showed that treatment with EC_{30} methylone for 2, 6 and 24 h increased the LC3-II/LC3-I ratio 14-, 15- and 25-fold over control values ($p<0.05$), respectively (Fig. 3A). For cells treated for 24 h with EC_{60} methylone or MDPV, the rises in LC3 turnover were significant for both substances, with a 47-fold increase for methylone ($p<0.0001$ *vs.* control) and 13-fold increase for MDPV ($p<0.01$ *vs.* control) (Fig. 3B).

The observed rise in LC3-II levels may be associated to either the increase of autophagosome formation in the early stage or the defective degradation in the late stage, which results in the accumulation of autophagic vacuoles. Therefore, to clarify the role of autophagosome accumulation, we employed Baf A1 to affect the autophagic flux and monitored LC3-I to LC3-II turnover. Baf A1 inhibits the fusion between autophagosomes and lysosomes (Yamamoto et al. 1998) and, thus, if autophagic flux is occurring, the level of LC3-II expression will be increased in the presence of this inhibitor, since the transit of LC3-II through the autophagic pathway will be blocked. The results showed that, as expected, Baf A1 treatment alone enhanced LC3-II levels in SH-SY5Y cells (Fig. 3C). More importantly, the levels of LC3-II in cells treated with Baf A1 and methylone/MDPV together are higher than that treated by Baf A1 alone. This suggests that β-KA increases LC3-II levels primarily through the enhancement of autophagosomal formation rather than through inhibition of autophagosomal degradation.

Figure 3

Further validation of autophagosome formation was assessed by electron microscopy analysis. As shown in Fig. 4, non-treated SH-SY5Y cells are characterized by regular contours and membrane projections, typical cytoplasmic appearance, and

absence of any abnormal feature worthy of consideration (Fig. 4A). In contrast, the ultrastructural analysis of cells exposed to methylone and MDPV revealed distinctive morphological features of autophagic process. For instance, cells incubated with EC_{60} MDPV for 24 h exhibit many vacuoles dispersed throughout the cytoplasm (Fig. 4B, red arrowheads), variable in shape and volume. The high-magnification image of a cell exposed to EC_{60} methylone for 6 h (Fig. 4C) shows that these vacuoles present a lamellar, vesicular structure surrounded by a double membrane (yellow arrowhead) and appear to engulf intracellular contents and whole organelles, including mitochondria (blue arrowhead), which are distinguishing morphological features of autophagosomes. Of note, in the most prolonged exposure time tested (24 h), methylone- and MDPV-treated cells also exhibited dark vacuoles with high-density single-membrane structures, which are consistent with autolysosome degradation of the enclosed materials. These results further support that the autophagic flux is increased in SH-SY5Y cells exposed to β-KA.

Figure 4

Autophagy precedes apoptosis and involves oxidative stress

There is a close crosstalk between the autophagic and apoptotic pathways, with common signaling factors maintaining a balance that regulates cytoplasmic contents and cellular turnover, respectively (Marino et al. 2014). Considering that methylone and MDPV trigger apoptosis in SH-SY5Y cells (Valente et al. 2017), we aimed at determining which mechanism takes place first. As depicted in Fig. 5, the markers of autophagy herein studied, namely LC3-I/LC3-II turnover (Fig. 5A) and formation of AVOs (Fig. 5B), are significantly increased at a concentration and time of exposure (EC_{30}, 2 h) at which apoptosis and the reduction in cell viability are absent, as suggested by caspase 3 activity (Fig. 5C) and MTT reduction measurements (Fig. 5D), respectively. This result was clearly more evident for methylone, for which the rise in LC3-II/LC3-I ratio and percentage of AO^+ events are accompanied by a significant increase in ROS and RNS production (Fig. 5E), suggesting an involvement of oxidative stress in the activation of autophagy.

Figure 5

N-acetyl-L-cysteine attenuates β-keto amphetamines-induced neurotoxicity by inhibiting autophagy and apoptosis

To assess the contribution of oxidative stress to autophagic activation, we evaluated the effects of the antioxidant NAC in the expression of LC3-II, caspase-3 activation, cell viability and ROS and RNS production in SH-SY5Y cells exposed to methylone and MDPV for 24 h. As depicted in Fig. 6, NAC, acting as a ROS scavenger, completely reversed the production of reactive species elicited by methylone and MDPV to control levels (Fig. 6A), while partially, but significantly, decreasing cell death induced by methylone and MDPV from approximately 60% to 47.8 ± 1.5 and 37.8 ± 2.0%, respectively (Fig. 6B). In addition, a visible decrease of LC3-II expression was observed in the presence of NAC ($p<0.05$) (Fig. 6C), indicating a decrease in autophagosome formation, and thus an inhibition of β-KA-induced autophagic activity. Furthermore, NAC was also able to reduce methylone- and MDPV-induced apoptosis, as confirmed by the significant decline in caspase 3 activation of about 68 and 73 %, respectively, in the presence of the antioxidant (Fig. 6D). Taken together, these results suggest that NAC attenuates β-KA-induced neurotoxicity through the partial blocking of both autophagy and apoptosis, with oxidative stress playing a key role in this regulation.

Figure 6

Discussion

Autophagy is a programed and evolutionarily-conserved catabolic process through which, at basal levels, cells digest intracellular contents in order to eliminate damaged organelles or unfolded proteins and maintain cellular homeostasis (Mizushima 2007). Autophagy involves the formation of double membrane-bound structures called autophagosomes that engulf cytosolic aged proteins and organelles, particularly mitochondria. Autophagosomes ultimately fuse with lysosomes to form single membrane autolysosomes, within which the cargo is degraded (He and Klionsky 2009). Under various cellular and environmental stress conditions, such as nutrient depletion and oxidative stress, autophagy can be induced to maintain or promote cell survival or, upon extended insult, may be detrimental and act as a mode of cell death, either by cellular atrophy and collapse of cellular functions (autophagic or type II cell death), or through the activation of apoptotic (or type I cell death) or necrotic (or type III) cell death, suggesting that autophagy plays a key role in controlling cell metabolism and fate (Galluzzi et al. 2008; He and Klionsky 2009; Jaeger and Wyss-Coray 2009).

Here, we provide the first evidence that β-KA are able to induce a robust autophagic response in dopaminergic neurons *in vitro*. Our findings showed that methylone and MDPV, two commonly abused β-KA derivatives, elicited morphological and biochemical changes consistent with autophagy and neurodegeneration. Both drugs prompted the formation of AVOs and increased expression of the autophagy-associated protein LC3-II in a concentration- and time-dependent manner. In accordance with these results, electron microscopy confirmed the increased autophagic flux in dopaminergic SH-SY5Y cells after drug exposure, as evidenced by the visualization of abundant double-membrane autophagosomes and autolysosomes. Bafilomycin A1 further demonstrated that LC3 turnover was primarily related to the enhancement of autophagosome formation/maturation rather than to the blockade of autophagosomes and lysosomes fusion. Additionally, we provide evidence that methylone- and MDPV-mediated induction of autophagy is an early event following drug exposure. Indeed, the autophagy markers (AVOs formation and LC3 turnover) were consistently positive in SH-SY5Y cells as early as 2 h after drug exposure. More importantly, our results indicate that, at this time point, no effects on the apoptotic marker (i.e. caspase-3 activation) or cell viability were observed, thereby indicating that autophagy may be an initial process that precedes apoptotic cell death.

Consistent with previous works (den Hollander et al. 2014; Rosas-Hernandez et al. 2016a; Valente et al. 2017), the present study showed that ROS are generated during neuronal exposure to β-KA, with this effect being more pronounced for methylone and already visible after 2 h of exposure. It is known that excessive accumulation of ROS may hamper the cellular homeostasis, leading to oxidative stress and mitochondrial dysfunction (Chakrabarti et al. 2014), and that basal autophagy enables the physiological turnover of damaged organelles, long-lived proteins, and cytoplasmic contents (He and Klionsky 2009). Under mild oxidative conditions that surpass the cell defenses, autophagy is upregulated as an adaptive stress response, degrading and recycling oxidized (damaged) biomolecules, whereas excessive oxidative stress may result in autophagic cell death (Navarro-Yepes et al. 2014). Recent studies support the role of ROS and RNS as intracellular signal transducers, triggering autophagosome formation and autophagic degradation, and thus, by disposing cells of oxidatively damaged proteins and organelles, autophagy helps reduce the elicited oxidative stress (Filomeni et al. 2015). In line with this, *in vitro* studies show that the generation of ROS is involved in methamphetamine-induced autophagy in neuronal cells (Chandramani Shivalingappa et al. 2012; Huang et al. 2017; Li et al. 2012). For instance, Huang et al. (2017) showed that 5 mM methamphetamine induces significant ROS generation in neuron-glial cells after 1 h of exposure and upregulation of autophagic biomarkers after 3 h, while cell

damage, as determined through the LDH leakage assay, was only evident after 6 h of treatment. A similar mechanism can be postulated for methylone and MDPV, activating autophagy to alleviate oxidative stress and protecting neuronal cells from apoptotic cell death. Supporting this hypothesis, we showed that, though methylone increased ROS levels 2 h after drug exposure, we were unable to detect any evidence of cell death or apoptosis activation, suggesting that ROS production induced by methylone (and to a less extent by MDPV) may play a role in cell survival at an initial stage rather than instigate a toxic effect.

The disruption of the intracellular redox status has been extensively implicated in the neurotoxicity of amphetamines, and involves several mechanisms including increased generation of ROS and RNS, oxidative deamination of released catecholamines, mitochondrial dysfunction, metabolism of the methylenedioxy ring, excitotoxicity, microglial activation, depletion of antioxidant systems and hyperthermia (Carvalho et al. 2012; Halpin et al. 2014). Due to the structural similarities of methylone and MDPV to these illicit substances, it may be postulated that synthetic cathinones elicit neuronal oxidative stress through mechanisms similar to those of amphetamines. For instance, direct oxidation of accumulated DA in the presynaptic cleft and subsequent formation of reactive DA quinones appears to contribute to the neuronal production of ROS by methamphetamine (LaVoie and Hastings 1999), and it may also be expected from synthetic cathinones that have the ability to decrease the reuptake of DA, which is the case of the derivatives herein studied. Furthermore, evidence shows that both methylone and MDPV undergo *O*-demethylenation of the methylenedioxy ring, analogous to MDMA, with formation of catechols (Pedersen et al. 2013; Strano-Rossi et al. 2010), which, for MDMA, are well-known precursors of the highly reactive and ROS generating *ortho*-quinones (Monks et al. 2004). Hyperthermia is another important factor contributing for the exacerbation of oxidative damage induced by amphetamines (Carvalho et al. 2012), and both methylone and MDPV were reported to elicit the rise in body temperature in humans (Barrios et al. 2016; Borek and Holstege 2012) and animal models (Anneken et al. 2015; King et al. 2014). Of note, we recently demonstrated the *in vitro* potentiation of MDPV-induced hepatotoxicity by hyperthermia, characterized by the increase in both oxidative stress and mitochondrial dysfunction markers (Valente et al. 2016b). Additionally, mitochondria are also a major source of ROS and recent studies support the ability of β-KA, including methylone and MDPV, to impair mitochondrial respiratory function *in vitro* (den Hollander et al. 2014; den Hollander et al. 2015; Nakagawa et al. 2009; Valente et al. 2016a; Valente et al. 2016b; Valente et al. 2017), which is characterized by marked ATP depletion, increased calcium levels and mitochondrial depolarization.

Our group has recently demonstrated that oxidative stress is involved in methylone- and MDPV-elicited neuronal cell death, eliciting the generation of ROS and RNS, decline in the intracellular levels of GSH and increased oxidized glutathione levels (Valente et al. 2017). NAC is a thiol-based antioxidant that acts as a ROS scavenger and as a precursor for GSH biosynthesis (Firuzi et al. 2011). Based on NAC's beneficial effects, we hypothesize that it may elicit a protective effect against β-KA-induced neurotoxicity by modulating oxidative damage. Previous studies showed that apoptosis is a common mechanism of cell death induced by methylone and MDPV, with evident DNA fragmentation, chromatin condensation and formation of pyknotic nuclei, and caspases activation (Adam et al. 2014; Rosas-Hernandez et al. 2016a; Valente et al. 2016a; Valente et al. 2016b; Valente et al. 2017). Therefore, we also examined the relationship between cellular redox status, autophagy, and apoptotic cell death following drug exposure. Our results revealed that NAC completely reversed the redox imbalance elicited by methylone and MDPV and, most importantly, partially attenuated both autophagic and apoptotic activity, as demonstrated respectively by the decrease in LC3 turnover and caspase-3 activity, which resulted in a significant decrease of cell death. ROS-mediated events may not be the sole redox-related event involved in the regulation of autophagy. Other factors, such as GSH redox status, have also been shown to regulate autophagy (Filomeni et al. 2010). In line with this, Chandramani Shivalingappa et al. (2012) showed that the activation of autophagy by methamphetamine in dopaminergic neurons was associated with the depletion of GSH, and NAC, acting as the precursor for the biosynthesis of GSH, significantly reduced autophagy and apoptosis in methamphetamine-treated cells, thereby exhibiting neuroprotective effects. Nonetheless, we have recently shown that the decrease in neuronal GSH levels elicited by methylone and MDPV in the present *in vitro* model is a later event, with ROS and RNS production taking place before GSH depletion (Valente et al. 2017), leading us to consider that NAC protective effects against β-KA neurotoxicity may be primarily related to its ROS scavenging properties. Altogether, our study highlights the central role of cellular redox status both in the mechanism of neuroprotection and modulation of autophagy.

There are several signaling pathways described in the literature by which ROS may regulate autophagy, including the blockade of the inhibitory pathway of the mechanistic target of rapamycin (mTOR) (Lee et al. 2012), and upregulation of several pro-autophagic genes, such as p53, p62, extracellular regulated kinase (ERK), hypoxia-inducible factor-1 and adenosine monophosphate-dependent protein kinase (AMPK) (Li et al. 2015; Scherz-Shouval and Elazar 2011). For instance, MDMA-induced neuronal autophagy appears to involve AMPK signaling, but not the inhibition of the mTOR

complex (Li et al. 2016; Li et al. 2014), whereas methamphetamine targets the mTOR and the ERK1/2 pathways (Ma et al. 2014). Further studies are underway to ascertain which molecular signaling pathways may be involved in autophagy triggered by β-KA and in its regulation by oxidative stress.

The crosstalk between autophagy and apoptosis is complex and decisive to cellular fate. Under certain cellular conditions, autophagy can promote cell survival and halt apoptosis, while in other circumstances, it may culminate in cell death, either independently or in close collaboration with apoptosis (Eisenberg-Lerner et al. 2009). The two mechanisms are regulated by common factors and each can regulate and modify the activity of one another (Marino et al. 2014). Our finding that NAC, acting as an antioxidant, can alleviate methylone- and MDPV-stimulated autophagy while rescuing cells from apoptotic cell death, further supports the role of autophagy as a cellular self-defence response against β-KA-induced oxidative stress and subsequent cell death.

Conclusions

To the best of our knowledge, this is the first report demonstrating that methylone and MDPV induce autophagy in human dopaminergic SH-SY5Y cells, thus constituting a new insight into the context of β-KA-related neurotoxicity. Our work also demonstrates that increased ROS levels is one of the pivotal mechanisms involved in β-KA-induced neurotoxicity and autophagy in dopaminergic SH-SY5Y cells. Furthermore, scavenging of free radicals such as ROS and RNS, using NAC, partially attenuated β-KA-induced upregulation of autophagy and apoptosis, leading to a significant reversion of the neuronal cell death elicited by these substances. These findings highlight the importance of cell redox status in β-KA-induced dopaminergic cell death and support the use of antioxidant agents to ameliorate the neurotoxic effects of these new psychoactive substances. Further studies will be necessary to confirm the effectiveness of NAC on the prevention of β-KA-induced dopaminergic degeneration *in vivo*.

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Figures

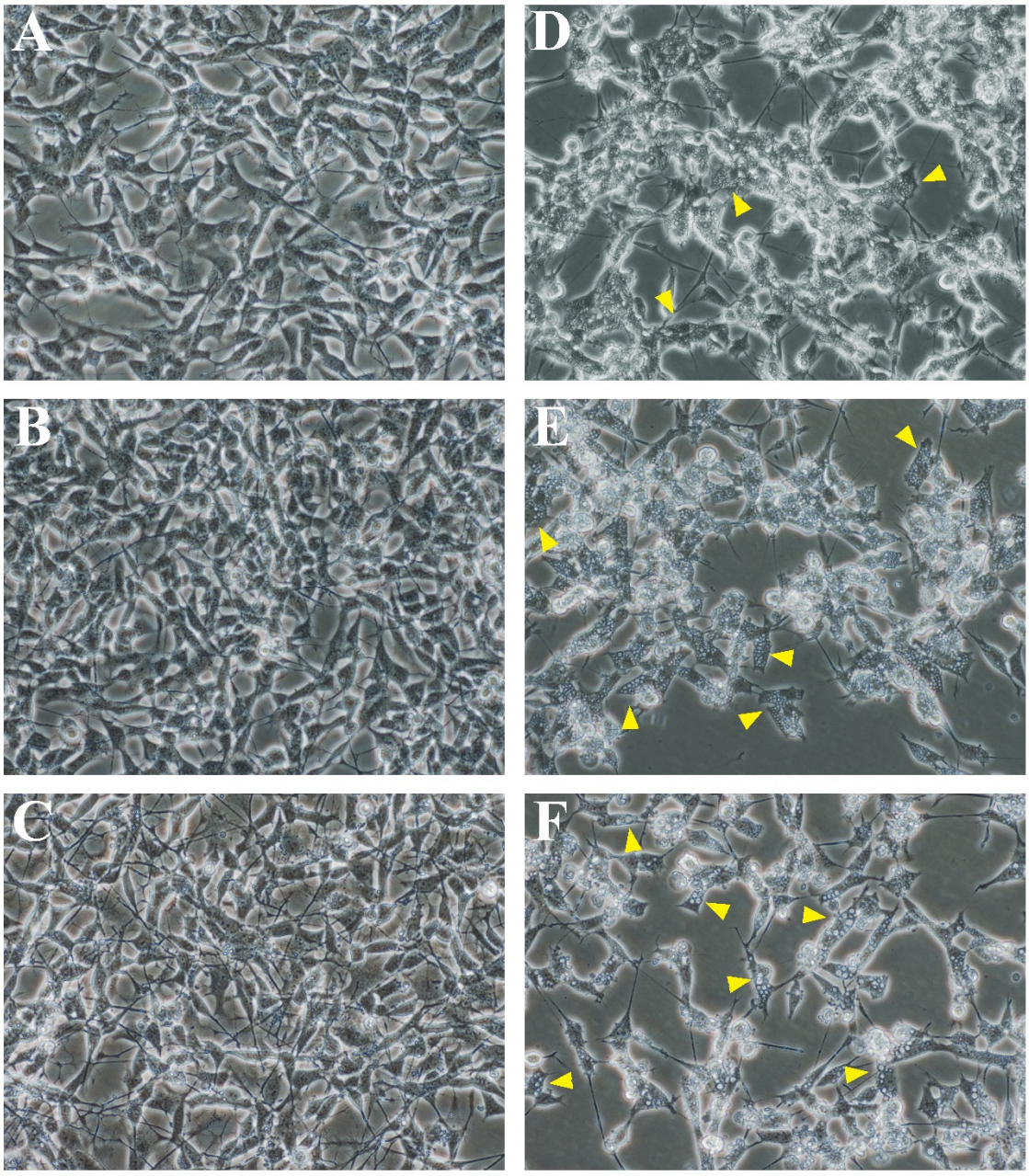

Fig. 1 Representative phase contrast images demonstrating morphological changes in dopaminergic SH-SY5Y cells exposed to a β-KA. Cells treated with EC_{60} methylone for 2 h (D), 6 h (E) and 24 h (F) exhibit a visible time-dependent neurite retraction and intracellular vacuoles increase in number and size (yellow arrowheads), when compared to control cells observed at the same time points (A – C)

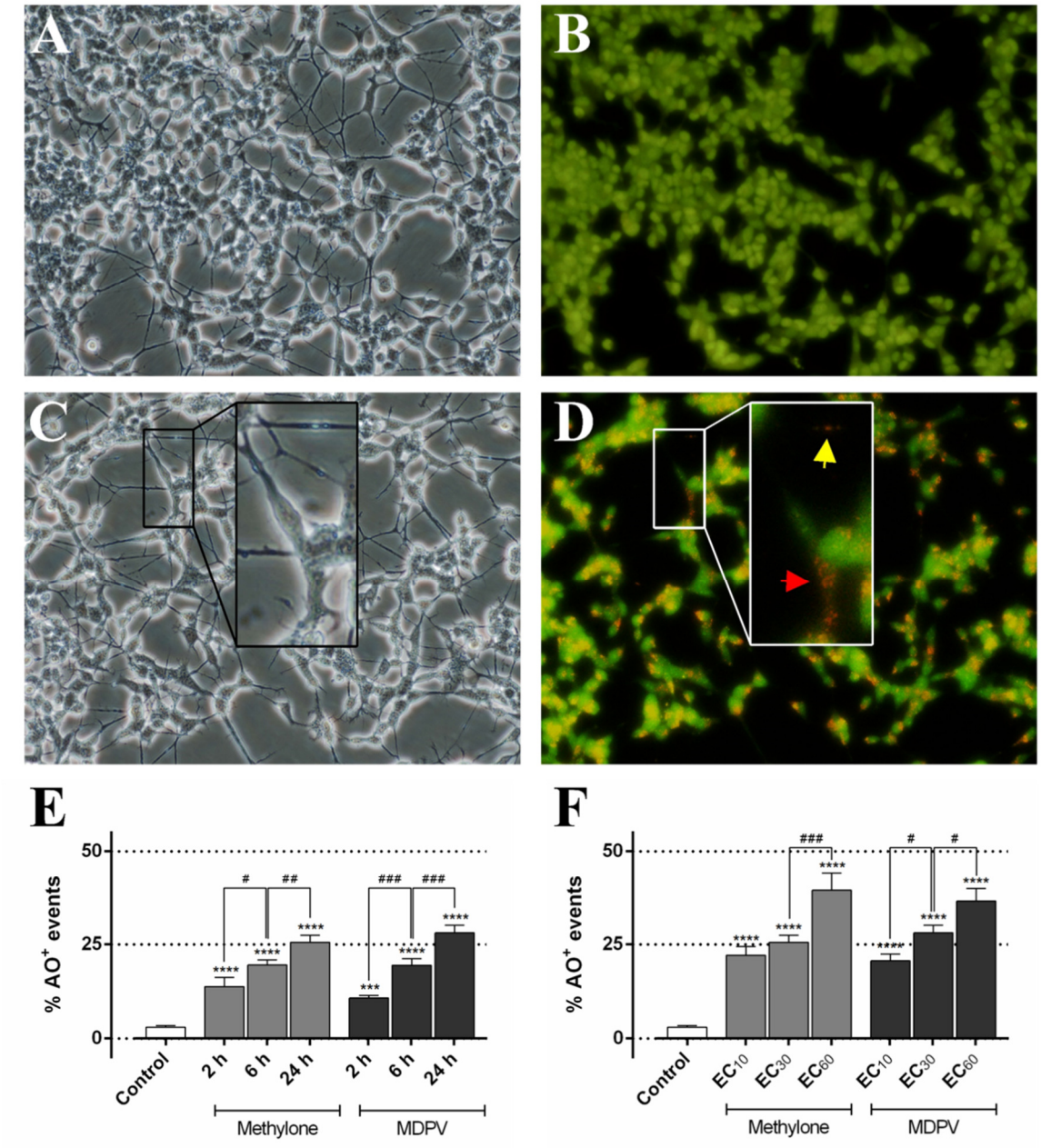

Fig. 2 Representative phase contrast (A and C) and fluorescence (B and D) micrographs of dopaminergic SH-SY5Y cells stained with acridine orange (AO) after exposure to a β-KA. Cells treated with EC60 MDPV for 6 h (C and D) present acidic vesicular organelles stained in red, appearing in both cell stroma (red arrow) and neurites (yellow arrow), which are scarce or absent in control cells (A and B). Quantification of AO^+ events in dopaminergic SH-SY5Y cells exposed to methylone and MDPV (E and F). Both compounds elicited a significant increase of AO^+ events in a time- (E; EC_{30}) and concentration-dependent manner (F; 24 h). $^{***}p<0.001$, $^{****}p<0.0001$ vs. control. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$

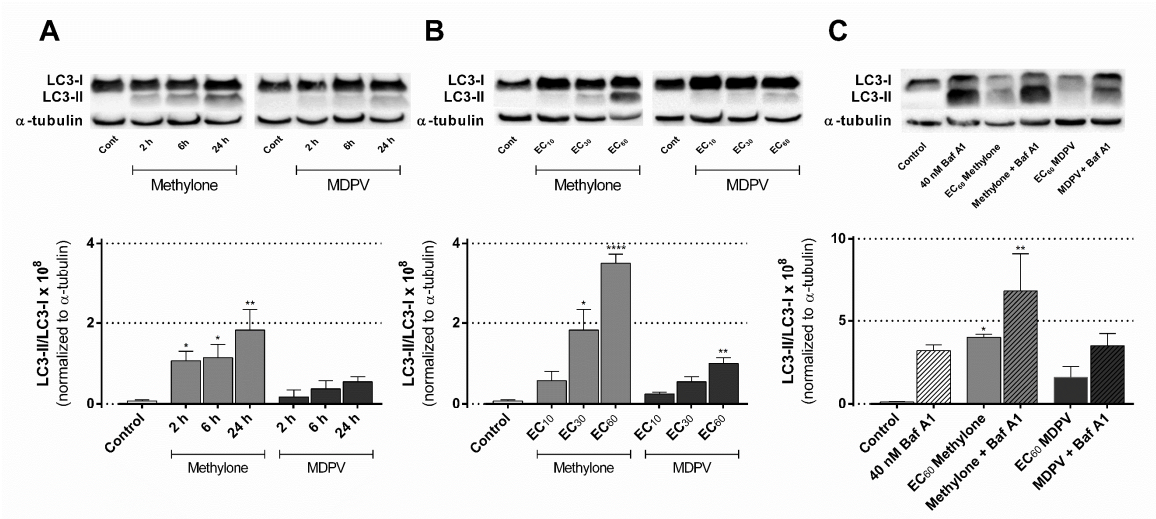

Fig. 3 Representative blots showing LC3I/LC3II and α-tubulin expression and LC3II/LC3I ratio in cells exposed to(A) EC_{30} methylone or MDPV for 2, 6 and 24 h, (B) EC_{10}, EC_{30} and EC_{60} methylone or MDPV for 24 h, and to (C) 40 nM Baf A1 and/or EC_{60} methylone or MDPV for 24 h. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. control

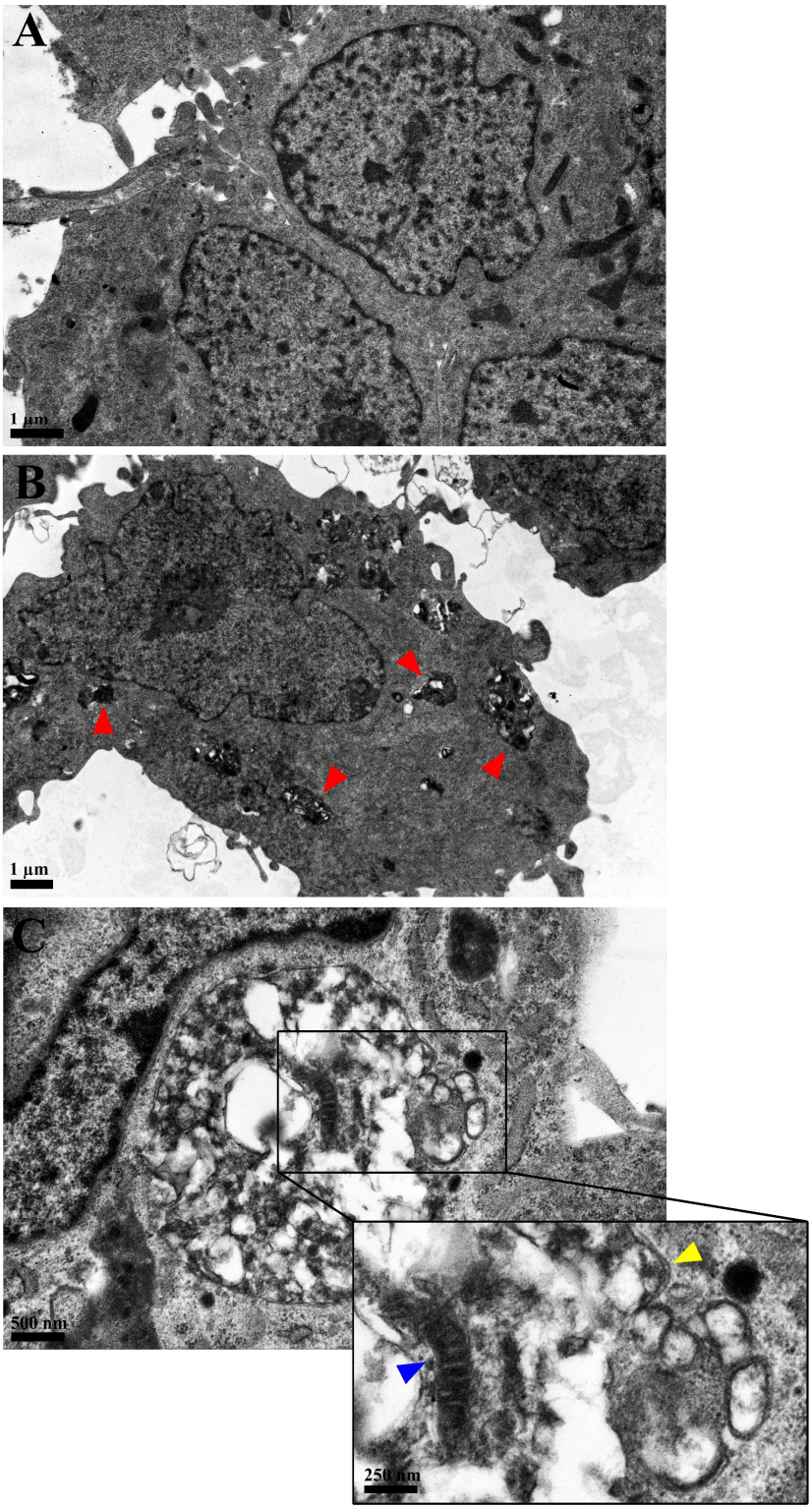

Fig. 4 Representative transmission electron micrographs demonstrating increased autophagic vacuoles in dopaminergic SH-SY5Y cells exposed to β-KA. Compared to control cells (A; 15 000 x magnification), cultures exposed to EC60 MDPV for 24 h (B; 12 000 x magnification) exhibit autophagic vacuoles (red arrowheads) scattered throughout the cytoplasm. These vacuoles, already present after 6 h of exposure to EC60 methylone (C; 30 000 x magnification), present double membrane (yellow arrowhead), characteristic of autophagosomes, which engulf large amounts of cellular content and even whole organelles, such as mitochondria (blue arrowhead)

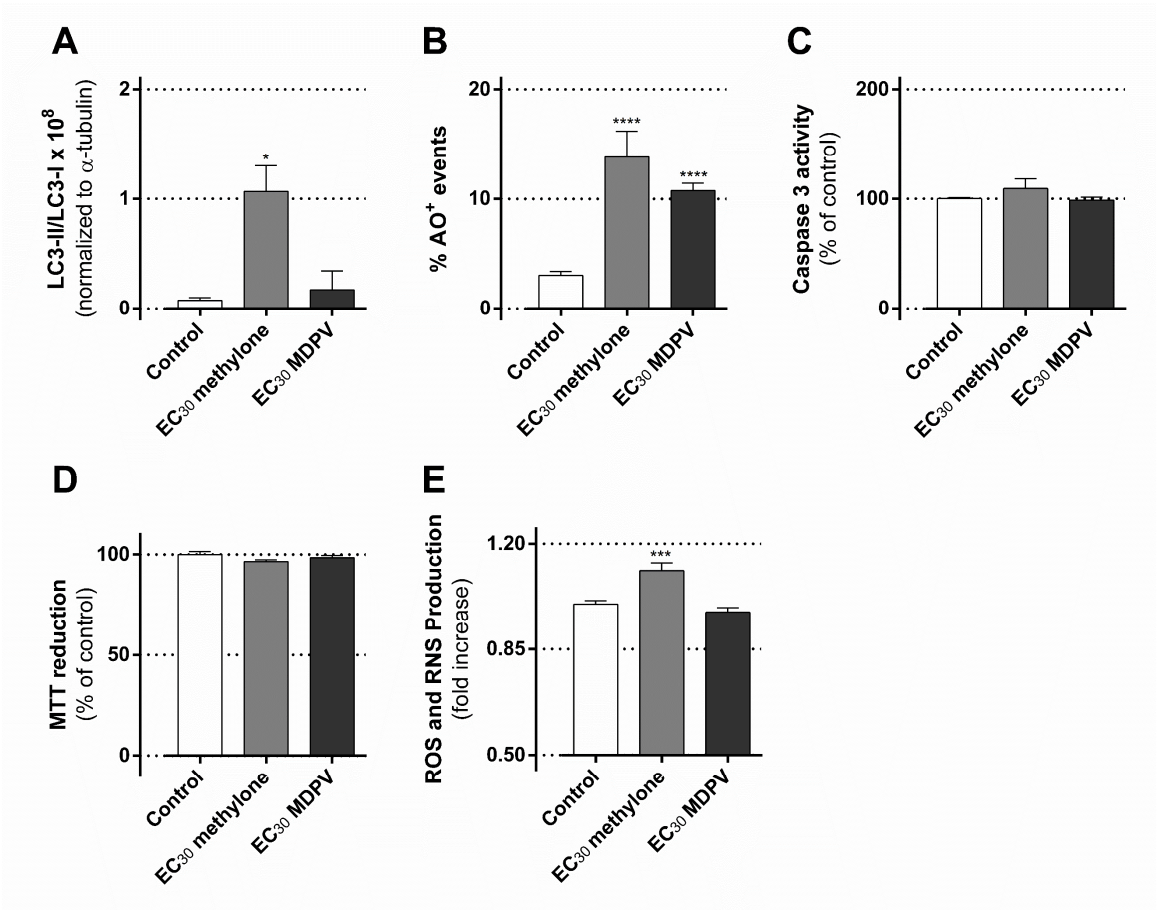

Fig. 5 (A) LC3II/LC3I ratio, (B) quantification of AO^+ events, (C) caspase 3 activation, (D) MTT reduction and (E) ROS and RNS production in cells exposed to EC_{30} methylone or MDPV for 2 h. *p<0.05, ***p<0.001, ****p<0.0001 vs. control

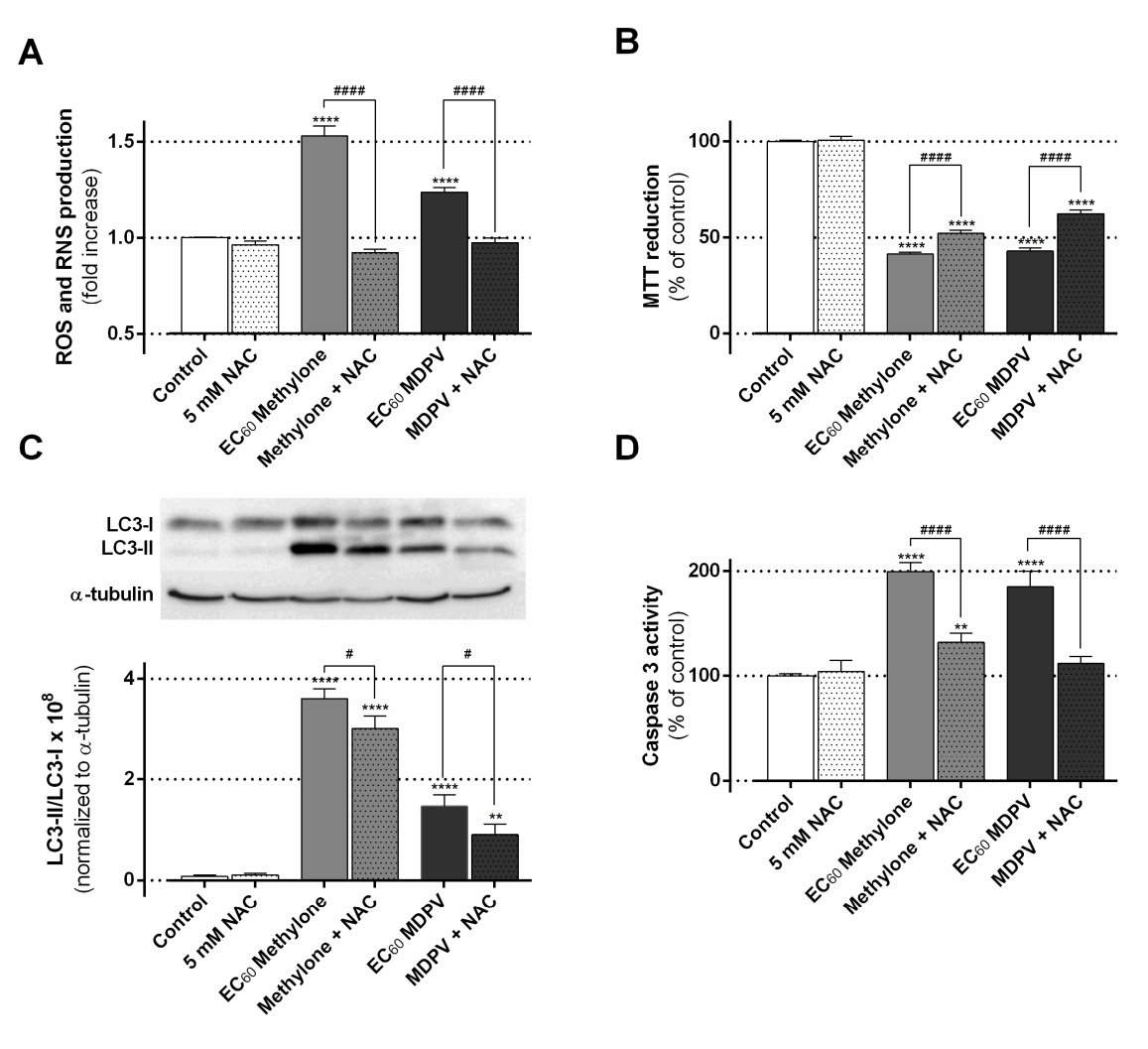

Fig. 6 Evaluation of (A) ROS and RNS production (B) MTT reduction, (C) LC3I/LC3II turnover (with representative blots) and (D) caspase 3 activation, after 24 h of incubation of dopaminergic SH-SY5Y cells with 5 mM NAC and/or EC_{60} methylone or MDPV. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ vs. control. $^{\#}p<0.05$, $^{\#\#\#\#}p<0.0001$

Section IV – Integrated discussion

The current knowledge on the potential harmful effects arising from the abuse of β-keto amphetamines is very limited, and aside from reported clinical complications and pharmacological modes of action, little is known about the mechanisms of cytotoxicity involved in the end-organ effects, in particular acute liver failure and CNS damage.

In the present thesis, we aimed at unveiling the cellular hepato- and neurotoxicity mechanisms prompted by β-keto amphetamines. Considering their chemical and pharmacological resemblance to non-keto amphetamines, comparative toxicological studies were conducted in the presence of MDMA, a widely recognized hepato- and neurotoxicant.

Experimental data incorporated in this thesis covered the assessment of mechanisms known to have an important role in the cytotoxicity mediated by classical amphetamines, namely oxidative stress, mitochondrial dysfunction, activation of programmed cell death pathways and hyperthermia. The obtained results were extensively discussed within each chapter from the previous section. In this section, the intent is to integrate all data and discuss the factors that may play a major role in the hepatotoxicity and neurotoxicity of β-keto amphetamines.

Characterization of the common mechanisms involved in the in vitro toxicity triggered by β-keto amphetamines in hepatic and neuronal cell models, and the relevance of structural modifications on the observed cytotoxicity

In the assessment of the hepatotoxic and neurotoxic potential of frequently abused cathinones, we found the activation of common mechanisms of oxidative stress, mitochondrial dysfunction and programmed cell death. Like MDMA, β-keto amphetamines elicited hepatic and neuronal cell death in a concentration-dependent manner, though with variable potencies among derivatives (Manuscripts I and III). As aforementioned, the lipophilicity of compounds may influence their toxicity by determining their ability to cross bilipid layers and reach target cells. In fact, we found a direct correlation between the predicted lipophilicity of the tested compounds and their hepatotoxic potential, i.e. the compound with lower log *P* value, methylone, was the least hepatotoxic drug, while MDPV and pentedrone, with log *P* values higher than MDMA, were the strongest hepatotoxicants in both *in vitro* models used, namely primary rat hepatocytes (PRH) and the human hepatoma cell line HepaRG (Manuscript I). Accordingly, methylone was also less toxic than MDMA and MDPV in neuronal cells (Manuscript III). These results are in line with what is

acknowledged from the chemical structure of β-keto amphetamines: 1) the ketone group greatly increases the polarity of synthetic cathinones, which decreases their permeability and consequently reduces their psychostimulant and toxic potential when comparing to their non-keto analogues; 2) the pyrrolidine ring has an opposing effect, conferring greater permeability to pyrovalerone derivatives (Valente et al. 2014).

The studied cathinones triggered hepatic and neuronal oxidative stress through mechanisms similar to long-studied amphetamines, namely with increased production of ROS and RNS and GSH depletion (Manuscripts I, II and III). Intriguingly, ROS and RNS production elicited by each compound diverged from their toxic potential. Therefore, in an attempt to explain the differences found among derivatives, we assessed their reducing potential and found that methylone, pentedrone and 4-MEC, which elicited significant ROS and RNS production in PRH at lower concentrations, were the derivatives with higher redox potential, while MDPV, one of the most hepatotoxic derivatives, exhibited lower reducing ability (Manuscript I). In dopaminergic SH-SY5Y cells, methylone also elicited more generation of ROS and RNS than MDPV (Manuscripts III and IV), while MDMA was unable to trigger this mechanism (Manuscript III). In agreement to these results, we showed that MDMA, contrarily to cathinones, has virtually no redox potential (Manuscript I), which is also in conformity with the work of den Hollander et al. (2014). As weak bases, synthetic cathinones exhibit higher electron-donor capacity than MDMA, which combined with their ability to undergo keto-enol tautomerization, may validate the elevated redox potential, while the molecular stabilization from the pyrrolidine ring might be counteracting this effect (Coppola and Mondola 2012b; Leffler et al. 2014).

Recent *in vivo* studies showed that 4-MMC induces neuronal lipid peroxidation with adaptative regulation of antioxidant enzymes, including catalase, superoxide dismutase, glutathione peroxidase and NO synthase (Budzynska et al. 2015; Ciudad-Roberts et al. 2016; Lopez-Arnau et al. 2015). In our *in vitro* models, β-keto amphetamines induced GSH depletion and consequent GSSG formation (Manuscripts I, II and III). As a major endogenous antioxidant barrier, GSH is responsible for, among other protective functions, the direct scavenging of reactive species in hepatic and neuronal cells (Bains and Shaw 1997; Chen et al. 2013). In this process, GSH is oxidized into GSSG, thus explaining the results observed in the present thesis (Manuscript I and III). Furthermore, Meyer et al. (2014) validated the formation of GSH conjugates with five methylenedioxy cathinones, including methylone and MDPV, hypothesizing a conjugation at the aromatic ring, which is also conceivable for non-methylenedioxy derivatives, such as pentedrone and 4-MEC. This GSH conjugation may aggravate the depletion of GSH without interfering with GSSG levels.

Our results also suggest a crosstalk between oxidative stress, mitochondrial dysfunction and dysregulation of cellular calcium homeostasis, which was supported by the

increase in intracellular Ca^{2+} levels in PRH exposed to MDPV (Manuscript II), depletion of ATP (Manuscripts I, II and III), Δψm disruption in dopaminergic cells (Manuscript III), and the activation of the mitochondria-dependent apoptotic pathway, substantiated by the activation of caspase 9 in both target cells (Manuscripts I, II and III). Accordingly, the work of Nakagawa et al. (2009) showed that methylone elicited significant Δψm dissipation in isolated rat hepatocytes, accompanied by concentration-dependent ATP depletion, membrane blebbing and cell death, while 4-MMC was shown to inhibit the mitochondrial respiration in neuronal cells (den Hollander et al. 2014). Mitochondrial impairment might also lead to the exacerbation of the oxidative damage through the formation of mitochondria-derived ROS (Brookes et al. 2004), but it remains unclear whether mitochondrial dysfunction is potentiating the oxidative stress triggered by these substances. Nonetheless, the contribution of the oxidative damage to the overall cytotoxicity of β-keto amphetamines was confirmed by the protective effects of the antioxidant agents AA (Manuscript I) and NAC (Manuscripts I and IV) against induced cell death, which may be acting as ROS and RNS scavengers, AA may regenerate other antioxidants, and NAC can enact the synthesis of GSH (Firuzi et al. 2011; Maxwell 1995).

As previously suggested for MDMA (Capela et al. 2013; Dias da Silva et al. 2013), β-keto amphetamines also seem to have targeted death receptors in PRH and dopaminergic neurons, leading to the activation of mitochondria-independent apoptosis, as ascertained by the activation of caspase 8 attained with all derivatives in both *in vitro* models (Manuscripts I, II and III). Regardless of the upstream pathway of programmed cell death being triggered, the signaling cascades of such pathways ultimately converge to activate common downstream mechanisms, specifically the effector caspase 3 or 7 (McIlwain et al. 2013). Accordingly, we found that β-keto amphetamines triggered the activation of caspase 3 in PRH and dopaminergic SH-SY5Y cells (Manuscripts I, II and III). In both models, we also observed cellular morphological changes consistent with apoptotic cell death, namely cell shrinkage, chromatin condensation and formation of pyknotic nuclei, with an apparent increase in apoptotic events and a progression from an early to a late stage apoptosis in a concentration-dependent manner (Manuscripts I, II and III). In PRH, it was also possible to observe the existence of necrotic events at higher concentrations of β-keto amphetamines (Manuscripts I and II), while in neuronal cells, they were infrequent (Manuscript III). These data suggest that necrosis may be a more preeminent mechanism of cell death in the liver.

Relevance of metabolic activation in β-keto amphetamines-induced hepatotoxicity

In our study on β-keto amphetamine-induced hepatotoxicity (Manuscript I), methylone, MDPV, pentedrone and 4-MEC were 3.2- to 5.2-fold less potent in the human

HepaRG cell line than in PRH, which may be related to the level of metabolic enzyme expression in each model. In fact, HepaRG is a hepatoma-derived cell line obtained from a poor cytochrome P450 (CYP) 2D6 metabolizer individual (Guillouzo et al. 2007). Importantly, CYP2D6 is the main phase I metabolizing enzyme of synthetic cathinones (Negreira et al. 2015; Pedersen et al. 2013), which explains the higher susceptibility of PRH to cell death induced by these substances. The co-incubation with the selective CYP2D6 inhibitor, quinidine, partially prevented the β-keto amphetamines-elicited cell death, which supports the hypothesis of CYP2D6-mediated bioactivation of synthetic cathinones, a mechanism fully accepted for amphetamine derivatives (Carmo et al. 2007; Carmo et al. 2006).

As previously mentioned, the metabolic bioactivation plays an important role in the expression of MDMA-induced hepatotoxicity (Carvalho et al. 2004b). In this process, the methylenedioxy ring is opened, resulting in the formation of catechol metabolites, which are further oxidized into highly reactive *ortho*-quinones and semiquinones, that may enter redox cycling with formation of ROS and RNS, thus contributing to the elicited oxidative damage (Carvalho et al. 2012). Although the formation of *ortho*-quinones has not been reported to date for β-keto amphetamines, the demethylenation process and formation of catechol metabolites was shown to occur both *in vitro* and *in vivo* (Kamata et al. 2006; Meyer et al. 2010a; Pedersen et al. 2013; Strano-Rossi et al. 2010), and recent evidence suggest that these metabolites exhibit higher cytotoxicity than their parent compounds (Wojcieszak et al. 2016). Taken together, these data support the *in vitro* hepatic bioactivation of synthetic cathinones and its potentiating effect towards cell death.

Role of hyperthermia on β-keto amphetamines-induced hepatotoxicity

A prominent acute toxic effect of β-keto amphetamines is hyperthermia (Barrios et al. 2016; Beck et al. 2015; Zona et al. 2016), and the rise in temperature has been shown to potentiate the toxicity induced by MDMA in liver (Carvalho et al. 2001b; Pontes et al. 2008c) and neuronal *in vitro* models (Capela et al. 2006).

For the evaluation of the role of hyperthermia in β-keto amphetamines-induced hepatotoxicity (Manuscript II), we chose MDPV, one of the most prevalent derivatives and known to induce robust increases in human body temperature up to 41.7 ºC (Borek and Holstege 2012; Frohlich et al. 2011; Kesha et al. 2013; Mugele et al. 2012; Murray et al. 2012). We found that the increase in incubation temperature from 37 to 40.5 ºC significantly aggravated the hepatotoxic potential of MDPV in PRH, and potentiated all oxidative stress and mitochondrial dysfunction markers herein studied. This included a significant enhancement of ROS and RNS generation, and GSH and ATP depletion, a substantial,

though not significant, increase in Ca^{2+} intracellular levels, and a precipitation of both intrinsic and extrinsic apoptotic cell death pathways, as ascertained by the activation of caspase 8 and 9, as well as caspase 3, at lower concentrations. In agreement with previous works (Santos-Marques et al. 2006; Skibba et al. 1989), we showed that hyperthermia *per se*, elicited a significant decrease in GSH levels in non-treated PRH. We also observed morphological changes in control cells under hyperthermic conditions, namely the presence of pyknotic nuclei and fragmented chromatin, consistent with early apoptotic cell death. Of note, activation of caspases induced by MDPV reached a peak at the second highest concentration tested (0.8 mM), suffering a decrease at the highest one (1.6 mM). Microscopical analysis with fluorescent nuclei dyes (Hoechst 33342 and propidium iodide) evidenced the increased prevalence of necrotic events under hyperthermia. These results suggest that the increase in temperature rendered cells more vulnerable to necrosis, which was also described in PRH and HepG2 cells exposed to MDMA (Dias da Silva et al. 2013; Pontes et al. 2008c). Taken together, our data suggest that the thermoregulation impairment elicited by synthetic cathinones most certainly contributes to increased liver damage, and it may also potentiate neurodegeneration.

There is a great number of mechanisms by which amphetamines may induce hyperthermia (Carvalho et al. 2012). However, apart from the inhibitory effects on α-adrenoceptors (Simmler et al. 2013a; Simmler et al. 2014), there is still a lack of experimental data on the pro-hyperthermic mechanisms activated by β-keto amphetamines.

Role of autophagy on β-keto amphetamines-induced neurotoxicity

Autophagy is a programmed catabolic process through which, at basal levels, cells digest intracellular contents to eliminate damaged organelles or proteins, and maintain cellular homeostasis (Mizushima 2007). In this process, the damaged cytoplasmic content is engulfed by doubled-membrane vacuoles, called autophagosomes, and redirected to lysosomes for degradation and recycling (He and Klionsky 2009).

In the last experimental work, we presented the first evidence that β-keto amphetamines are able to induce neuronal autophagy (Manuscript IV). Our data showed that methylone and MDPV trigger the formation of AVOs in dopaminergic SH-SY5Y cells, and induce neurite retraction, two hallmark mechanisms of autophagy-related neurodegeneration (Chu et al. 2009; Paglin et al. 2001). Cells exposed to these two β-keto amphetamines also exhibited morphological and biochemical changes consistent with autophagic activation, which includes the instigation of autophagosome formation, with evident progression to autolysosomes, and increased expression of the autophagy-related protein LC3-II in a concentration- and time-dependent manner.

Under stress conditions, autophagy is known to be upregulated as an adaptive mechanism to promote cell survival. At extreme circumstances, it may be detrimental, acting as a programmed cell death pathway (autophagic cell death), or by enacting cell death by apoptosis or necrosis (Galluzzi et al. 2008). A growing number of *in vitro* studies support the ability of MDMA and METH to trigger autophagy in neuronal cells (Chandramani Shivalingappa et al. 2012; Kanthasamy et al. 2006; Larsen et al. 2002; Li et al. 2016; Li et al. 2014; Li et al. 2012; Mercer et al. 2017), however, the influence of autophagic activation on amphetamines-induced neurotoxicity remains a debatable matter, with evidence supporting either a neuroprotective role for autophagy (Mercer et al. 2017), or a mechanism of cell death potentiation (Li et al. 2016). Our data suggest that β-keto amphetamines-induced autophagic activation is an early neuronal event, with substantial upregulation of the autophagy markers (LC3 turnover and AVOs formation) already after 2 h of exposure. These effects also preceded the induction of cell death and caspase 3 activation, supporting the activation of autophagy prior to apoptosis, which is in line with other *in vitro* studies regarding METH-induced neurodegeneration (Huang et al. 2017; Kanthasamy et al. 2006; Ma et al. 2014). Interestingly, experimental evidence supports a complex interplay between the two programmed cell death pathways (Boya et al. 2005; Eisenberg-Lerner et al. 2009; Gonzalez-Polo et al. 2005; Yu et al. 2004), but further studies are required to ascertain the role of β-keto amphetamines-induced autophagy on neuronal apoptotic cell death.

Finally, considering the demonstrated potential of β-keto amphetamines to disrupt the neuronal redox balance and the key role of ROS on the regulation of autophagic activity (Li et al. 2015), we assessed the effects of NAC in the neurotoxicity elicited by methylone and MDPV, and particularly in the modulation of oxidative stress, autophagy and apoptosis. Our results support a neuroprotective role for this thiol-based antioxidant agent, eliciting a significant reversion of drug-induced cell death and complete inhibition of ROS and RNS production. Importantly, NAC partially inhibited methylone- and MDPV-elicited autophagy while reducing cell death by apoptosis, which highlights the central role of cellular redox status in the modulation of autophagy and neuroprotection.

A general overview of the putative mechanistic pathways triggered by β-keto amphetamines in hepatic and neuronal cells is presented in figure 4.

Notwithstanding the usefulness of *in vitro* data for the comprehension of toxicity mechanisms at the cellular, biochemical, and molecular levels, *in vitro* models have several limitations, including the absence of biokinetics or interaction with other systems and organs, making it difficult to mimic *in vivo* exposure. This may lead to the misinterpretation of acquired data and an erroneous extrapolation of the effects to the human. Therefore, the requirement of *in vivo* studies should never be underestimated.

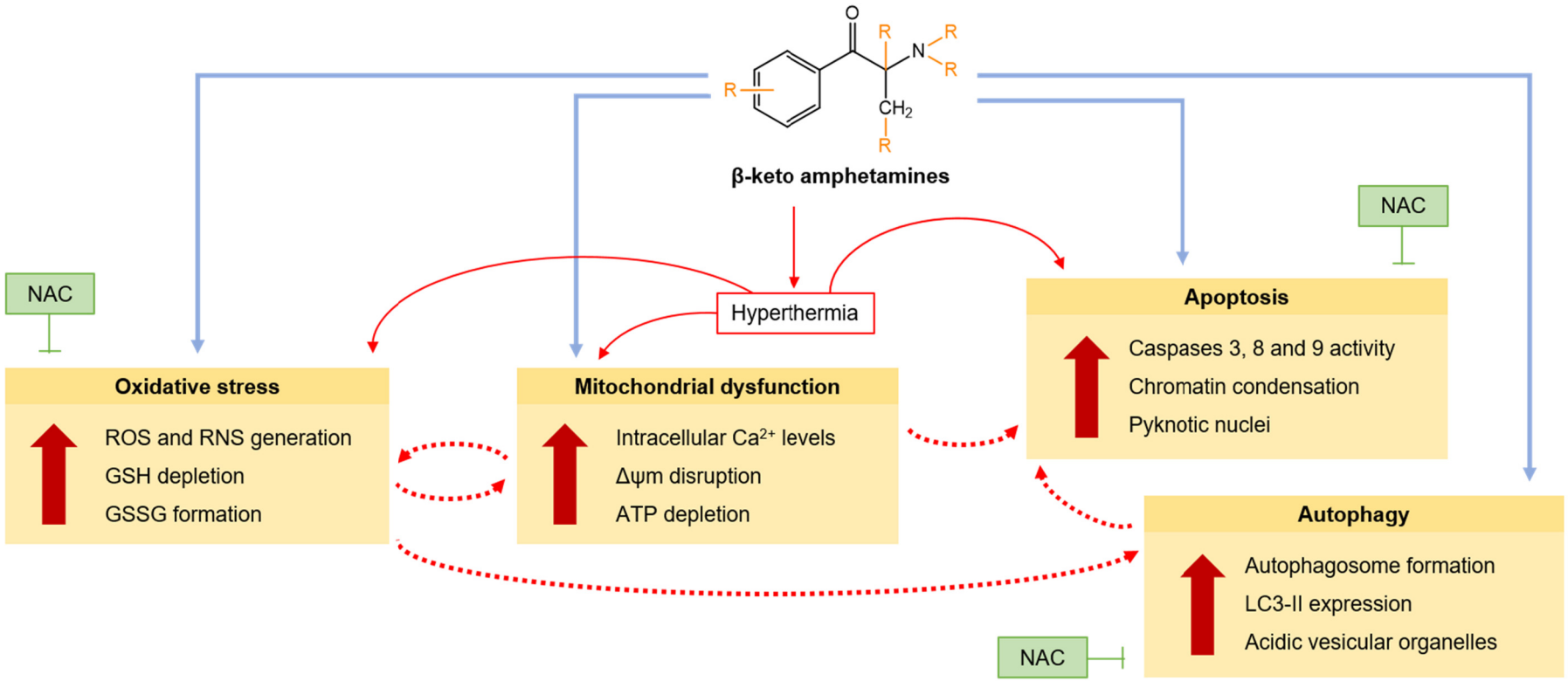

Figure 4: Schematic representation of putative mechanistic pathways triggered by β-keto amphetamines in hepatic and/or neuronal cells. *Common mechanisms of cytotoxicity in both target cells include the triggering of oxidative stress, with increased ROS and RNS generation, depletion of GSH and formation of GSSG, as well as impairment of mitochondrial functions, accompanied by the disruption of calcium homeostasis, and characterized by ΔΨm dissipation and ATP depletion. β-keto amphetamines also elicit cell death by apoptosis in both hepatic and neuronal cell models, characterized by evident chromatin condensation, presence of pyknotic nuclei and activation of caspases 3, 8 and 9, enacting the activation of both intrinsic and extrinsic pathways of apoptotic cell death. Autophagy was also determined to be involved in β-keto amphetamine-induced neurotoxicity, with an apparent crosstalk between oxidative stress, autophagy and apoptosis. Hyperthermia was shown to exacerbate the hepatotoxicity of MDPV, aggravating oxidative stress and mitochondrial effects and hastening the apoptotic cell death. NAC exhibited beneficial effects against the neurotoxicity elicited by methylone and MDPV, by restoring the cellular redox balance, and hampering both autophagic and apoptotic activation.*

Section V – Final conclusions and perspectives for future work

The experimental work conducted under the scope of this thesis has unveiled pivotal mechanisms underlying the hepatotoxicity and neurotoxicity of β-keto amphetamines. Overall, data presented herein allowed us to conclude the following:

- β-keto amphetamines trigger *in vitro* hepatotoxicity and neurotoxicity with a potency comparable to the classical amphetamine derivative MDMA
- The common mechanisms of cytotoxicity in hepatic and neuronal cells include the imbalance of the cellular redox status and consequent oxidative damage, and the impairment of mitochondrial functions
- The potential of each β-keto amphetamine derivative to induce oxidative stress and cell death appears to be correlated with their chemical properties, namely their lipophilicity/permeability and electron-donor capacity
- β-keto amphetamines activate both mitochondria-dependent (intrinsic) and mitochondria-independent (extrinsic) pathways of apoptotic cell death, in both hepatic and neuronal cells
- Hepatic metabolic bioactivation of β-keto amphetamines may be involved in the hepatotoxicity of these substances
- Hyperthermia exacerbates the hepatotoxic effects of the derivative MDPV
- β-keto amphetamine-induced toxicity in neuronal cells involves the activation of autophagy
- The modulation of oxidative stress by means of an antioxidant agent, such as NAC, may be beneficial in the treatment of β-keto amphetamine-related intoxications due to its ability to regulate both programmed cell death pathways, apoptosis and autophagy

Nonetheless, future work is required to attain full understanding of the mechanism of action of these drugs, to ascertain the relevance of the present *in vitro* findings to the overall adverse effects following β-keto amphetamine abuse, and to aid in the development of a comprehensive treatment approach of β-keto amphetamine-related intoxications. Future perspectives may potentially involve the following work:

- To clarify the relevance of metabolic bioactivation in β-keto amphetamines-induced cytotoxicity through the assessment of the cellular toxic potential of endogenously generated metabolites
- To elucidate the mechanisms involved in the altered thermoregulation reported for β-keto amphetamines
- To evaluate the toxic effects of β-keto amphetamines and respective metabolites in other target organs, such as the heart and kidneys, as well as the potential for eliciting BBB dysfunction
- To explore the role of autophagy in β-keto amphetamine-elicited toxicity, identifying the signaling pathways involved in autophagic activation, and finally to clarify the crosstalk between oxidative stress, autophagy and apoptosis elicited by these substances
- To validate the significance of *in vitro* data presented in this thesis through *in vivo* studies

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